Pregnane X Receptor (PXR) modulates NLRP3 Inflammasome

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master thesis

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Abstract:

Compounds released from the intestinal microbiota may play a role in maintaining mucosal homeostasis, but little is known about the receptors that sense and respond to these compounds. Recently, a cytosolic xenobiotic sensor, the pregnane X receptor (PXR), was identified as a receptor for microbial metabolites in the gastrointestinal (GI) tract. The PXR has been shown to play a protective role in the gut, with gene variants associated with IBD risk. Recent data suggest that the PXR may regulate innate immune signaling platforms, in a variety of tissues. In vascular endothelial cells, the PXR was shown to stimulate the expression of NLRP3, and initiate NLRP3 inflammasome activation. Interestingly, alterations in NLRP3 inflammasome function have been linked to IBD susceptibility. In the current thesis, we sought to characterize the role of the PXR in modulating the function of the NLRP3 inflammasome in macrophages, a key innate immune cell that contributes to host-defense and the regulation of intestinal mucosal homeostasis. Using the THP-1 cell line and mouse peritoneal macrophages, we found that PXR agonists stimulated caspase-1 activation, along with IL-1β processing and release. These responses were lost in cells lacking NLRP3 and blocked by selective inhibition of caspase-1. Furthermore, PXR-deficient cells failed to activate caspase-1 and release IL-1β in response to PXR agonist stimulation. Lastly, we found that PXR activation triggered ATP release, an effect that was responsible for inflammasome activation, as these responses were abolished by apyrase and P2X7 inhibition. Through this thesis, we demonstrated that the PXR activates the NLRP3 inflammasome through stimulating ATP release within a macrophage.
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<th>Description</th>
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<tbody>
<tr>
<td>acetaminophen</td>
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<td>pregnane x receptor</td>
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<td>vitamin D receptor</td>
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Chapter One: Introduction

1.1 Immune Systems within the Gastrointestinal Tract

The gastrointestinal (GI) tract relies on many interlinking aspects to allow for proper digestion, uptake of nutrients, and immune homeostasis (Bernstein et al., 2006). In order to preserve intestinal integrity, pathogens and other noxious luminal contents must be effectively eliminated; two mechanisms, the innate and adaptive immune systems, ensure the removal of harmful microorganisms and pathogens (Chen et al., 2009). While the adaptive immune system encompasses highly specialized and diverse factors accumulated through past exposure to antigens, the innate immune system concentrates solely on the first line of defense against pathogens. As a rapid response to noxious elements, the innate immune system monitors the surrounding environment and invokes a non-specific reaction through several effectors including dendritic cells, epithelial cells, and macrophages among others (Chen et al., 2009) (Abraham and Medzhitov, 2011).

One mechanism commonly utilized by the innate immune system in response to pathogens is inflammation (Baroja-Mazo et al., 2014). This component of the innate immune system can be triggered by pathogen-associated molecular patterns (PAMPS), danger-associated molecular patterns (DAMPS), stressed-induced, or via pathogen recognition receptors (PRRs). Once activated, PRRs stimulate downstream signaling pathway that contribute to the activation of inflammatory caspases and the production of a variety of pro-inflammatory mediators (Guo et al., 2015). Ideally, the inflammatory response deals with the insult by eradicating the offending agent and simultaneously removes damaged tissue to allow for proper repair and tissue growth. However, a delicate balance is required; an insufficient inflammatory response will allow for the survival of invading pathogens and a failure to clear damaged cells, yet an overly aggressive
response can elicit tissue damage and potentially trigger chronic inflammation and that can contribute to the onset of inflammatory diseases (Baroja-Mazo et al., 2014) (Guo et al., 2015).

Dysregulated intestinal inflammation triggering prolonged damage has been shown to be characteristic of the inflammatory bowel diseases (IBD) (Xavier and Podolsky, 2007). Encompassing both Crohn’s Disease (CD) and ulcerative colitis (UC), IBD affects approximately 1 in 180 Canadians and pathogenesis is thought involve alterations in immune function, genetics, and exposure to environmental factors (Loftus, 2004) (Abraham and Medzhitov, 2011). While the intestinal mucosal immune system ideally maintains a balance between gut barrier protection and immune tolerance, a loss of this equilibrium is thought to contribute to the pathogenesis of IBD (Abraham and Medzhitov, 2011). Given its prevalence and severity of symptoms, novel strategies must be employed to predict and treat inflammatory diseases.

1.2 Innate immune system dysfunction and IBD

In response to evidence implicating alterations in the function of the immune system in the pathogenesis of IBD, recent studies have explored the role of innate immune dysfunction and disease susceptibility (van Lierop et al., 2013). As mentioned previously innate immune responses are triggered by microbial invaders or endogenous molecules, and in response initiates distinct host systems (Inohara et al., 2003). These systems include membrane-bound toll like receptors (TLRs) as well as proteins within the cytoplasm, namely nucleotide-binding domain (NBD) and leucine-rich repeat (LRR) proteins. These unique host systems can recognize conserved microbial molecular patterns, such as peptidoglycan (PGN) found in the cell wall in Gram-negative and Gram-positive bacteria, as well as lipopolysaccharide (LPS) from Gram negative cell walls. One such sensor of microbial patterns implicated in innate immune
dysfunction is nucleotide-binding oligomerization domain (NOD) 2. NOD2 is a member of the NLR (nucleotide binding domain, leucine rich repeat gene containing) protein family and acts as an intracellular receptor PRR that responds to microbial muramyl dipeptide (MDP) to trigger the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and evoke autophagy (van Lierop et al., 2013). Expression of NOD2 is generally found in monocytes, and has been implicating in the induction of inflammation (Ogura et al., 2001). The accepted NOD2 mechanism of action involves, firstly, upon binding to MDP, NOD2 oligomerizes and binds to caspase recruitment domain (CARD)- containing serine/threonine kinase RIP2. This initiates RIP2 oligomerization, stimulating the IKK complex to activate NF-κB signalling pathway inflammation (Ogura et al., 2001) (Abbott et al., 2004). Activation of the NF-κB pathway then triggers several pro-inflammatory, anti-inflammatory cytokines and defensins, including IL-1β. (Ogura et al., 2001) (Abbott et al., 2004) (McDonald et al., 2005).

The first link to innate immune dysfunction and IBD risk was revealed upon the discovery that mutations in the NOD2 gene were associated with increased risk for developing CD (Hampe et al., 2001) (Ogura et al., 2001). One of the first studies investigating the relationship between NOD2 mutations and IBD sequenced NOD2 gene coding regions and genotyped an insertion polymorphism of the leucine-rich region (LRR) in 512 individuals with IBD (Hampe et al., 2001). Their findings implicate a NOD2 gene insertion mutation as conferring susceptibility to CD but not UC. Furthermore, an alternate study by Hugot and others (2001) found that the NOD2 gene product potentially through inappropriate activation of NF-κB within monocytes, thereby conferring susceptibility to CD (Hugot et al., 2001). Examining variations in NOD2 gene within CD patients, this study posits that the highest, consistent variants were found within the LRR and adjacent regions. This suggests that these CD-linked
variants may impair recognition of microbial molecules via NOD2 or potentially prohibit NOD2 oligomerization (Hugot et al., 2001).

Multiple reports have suggested that NOD2 mutations are associated with defective IL-1β output and reduced innate immune system responsiveness; overall, consensus between unique investigations can be found in the identification of the innate immune system as a key regulator of gut homeostasis (van Lierop et al., 2013) (Hirota et al., 2011) (Villani et al., 2009). One such study performed by Maeda and others (2005) examined NOD2 mutations and its effects on NF-κB activity and IL-1β release via a variant to the mouse NOD2 locus (Maeda et al., 2005). NOD2 mutant mice displayed elevated NF-κB activation when exposed to MDP and more effectual processing and secretion of IL-1β (Maeda et al., 2005). Therefore, the innate immune system balance must be preserved to prevent chronic activation of the mucosal immune system, as seen in IBD.

Interactions between innate immune cells and surrounding environment directly affect gut homeostasis within the GI tract. Although highly complex and poorly understood, what is known is that abnormalities within the intestinal innate immune system are highly involved in IBD pathogenesis; however, it is commonly thought that defects in the innate immune defenses resulting in a hypofunctional mucosal immune system are associated with IBD pathogenesis (Siegmund, 2010). What can be conclusively stated is that further investigations are necessary to elucidate the role of the innate immune system within the healthy gut, and ultimately, within a dysfunctional intestinal model.
1.3 The Inflammasome

Inflammasomes, defined as multiprotein platforms of cytosolic protein complexes, are formed in response to microbial damage or cellular insult and consequently, initiate an innate immune response through activation of caspase-1 and downstream cleavage of pro-IL-1β and pro-IL-18, into their active forms. In its mature, active form, IL-1β is a pro-inflammatory cytokine that can be secreted from monocytic cells within the lamina propria, but is also found in tissue macrophages, natural killer cells, B lymphocytes, and dendritic cells (Harrison and Maloy, 2011) (Dinarello, 2009). Once activated, IL-1β can then trigger activation of myeloid cells and neutrophil migration into damaged tissue (Dinarello, 2009) (Harrison and Maloy, 2011). Caspase-1 is also shown to trigger IL-18 maturation and subsequent activation, which is involved in signalling in the intestinal epithelium to maintain epithelial integrity (Siegmund, 2010). Upon activation, IL-1β and IL-18 are released from the cell and drive inflammation in response to endogenous or exogenous danger signals (Peeters et al., 2015). Furthermore, in some cases cleaved caspase-1 has been shown to lead to pyroptosis, a conserved and discrete mechanism of programmed cell death (Dupaul-Chicoine et al., 2010).

Up to now, unique PRRs have been implicated in inflammasome formation – including, NLRP1, NLRP3, NLRC4, Pyrin, and AIM1 (He et al., 2016). Classically expressed in myeloid cells among other cell types and a key factor in innate immunity, all inflammasomes must have a PRR that acts as a sensor, but several variations in inflammasome form have been identified. Some commonalities seen include a caspase-recruitment domain (CARD), apoptosis speck-like protein (ASC), and cysteine protease caspase-1 (He et al., 2016).

Perhaps most well-known of the inflammasomes is the NLR family pyrin domain containing 3 (NLRP3) inflammasome, first discovered in 2002 when the late Dr. Jurg Tschopp’s
group demonstrated that aberrant IL-1β processing and release was seen in periodic fever syndromes was caused by mutations of NLRP3 inflammasome (Martinon et al., 2002). Subsequent to this, the same group discovered that NLRP3 activation was not limited to sensing microbial molecules, but also via danger signals, as seen in sterile inflammation (Martinon et al., 2006). His work not only initiated in-depth investigations into the mechanisms underlying inflammasome activation but also led to a successfully therapeutic approach to periodic fever syndromes in the form of anti-IL1 receptor antagonists (Martinon et al., 2002) (Martinon et al., 2006) (Dostert et al., 2008) (Dagenais et al., 2011). He is also credited for discovering key inflammasome activators including muramyl dipeptide (MDP), monosodium urate crystals (MSU), asbestos, silica, and viral DNA (Martinon et al., 2006) (Dostert et al., 2008).

The NLRP3 inflammasome has been shown to promote host immune defense once activated; for example, influenza A virus and Staphylococcus aureus are two among several pathogens this inflammasome has been demonstrated to be protective against (Franchi et al., 2012). To explore the role of host defense, one investigation performed by Witzenrath and others (2011) looked at the function of NLRP3 inflammasome activation in a S. pneumoniae lung infection model (Witzenrath et al., 2011). As a major cause of pneumonia and meningitis, S. pneumoniae was shown to activate NLRP3 and their findings suggest that NLRP3 provided a protective innate immune response, as NLRP3−/− mice experienced higher bacterial loads and increased mortality when exposed to S. pneumoniae (Witzenrath et al., 2011). To add to this, an alternate study conducted by Hise et al. (2009) used mouse macrophages exposed to Candida albicans, a fungal pathogen that can be fatal to immunocompromised individuals, to investigate the role of NLRP3 inflammasome and proinflammatory cytokine IL-1β in host-defense (Hise et al., 2009). Their discoveries showed that C. albicans stimulates IL-1β release, dependent on
NLRP3 inflammasome activation and that IL-1 receptor knockout mice displayed increased fungal burdens and increased mortality compared to their wild-type counterparts (Hise et al., 2009). Taken together, these data demonstrated that once activated, the NLRP3 inflammasome produces IL-1β which ultimately induces anti-bacterial, anti-viral, and anti-fungal defence to protect the host against pathogens (Witzenrath et al., 2011) (Hise et al., 2009) (Franchi et al., 2012).

Inflammasome activation, release of pro-inflammatory cytokines, and potentially subsequent pyroptosis are key stages of host defense against bacterial pathogens (Vladimer et al., 2013). Yet, while NLRP3 inflammasome activation and subsequent pyroptotic cell death can lead to infection clearance and repair, it can also trigger dysfunction that is responsible for several human inflammatory diseases (Rathinam et al., 2012) (Vladimer et al., 2013). Inflammatory diseases linked to NLRP3 inflammasome include a gain of function mutation in the NACHT domain of NLRP3 responsible for three separate disorders: familial cold autoinflammatory syndrome (FCAS), Muckle–Wells syndrome (MWS) and neonatal onset multi-systemic inflammatory disease/chronic infantile neurological cutaneous articular syndrome (NOMID/CINCA) (Menu and Vince, 2011). Together, these comprise the cryopyrin- associated periodic syndromes (CAPS) which have varying levels of disease severity and symptoms including rash, persistent fever, amyloidosis, and in some cases, neurological complications. These groups of disorders stem from point mutations resulting in constitutive activation of the NLRP3 inflammasome, resulting in constant IL-1β release without external stimuli (Agostini et al., 2004). Therapeutic targets of CAPS can be found in anti-IL-1β therapy, which has proven beneficial even in the severest form of CAPS, NOMID/CINCA (Cook et al., 2010) (Goldbach-Mansky et al., 2006) (Agostini et al., 2004).
With respect to the GI tract, studies have demonstrated a correlation between several single nucleotide polymorphisms (SNPs) within the non-coding region of the NLRP3 gene region and an increased risk of developing CD (Villani et al., 2009). Several studies suggest that NLRP3−/−, ASC−/− and caspase-1−/− mice are more susceptible to DSS-induced colitis and exhibit increased mortality (Hirota et al., 2010) (Zaki et al., 2010) (Dupaul-Chicoine et al., 2010). Together, these data suggest that response to microbial or endogenous molecules is, to some degree, mediated by NLRP3 inflammasome to maintain intestinal homeostasis. Additionally, SNPs in the non-coding region of NLRP3 gene have been associated with loss-of-function, resulting in decreased NLRP3 expression, further suggesting that the NLRP3 inflammasome may play a protective role in intestinal inflammatory diseases (Villani et al., 2009) (Menu and Vince, 2011). Overall, dysregulated inflammasome activation has been associated with numerous human heritable and acquired diseases; because of this, the NLRP3 inflammasome should be investigated to better understand how inflammasomes regulate immune responses.

1.4 NLRP3 inflammasome Mechanism of Action

First shown to be stimulated by DAMPS, including adenosine triphosphate (ATP) and monosodium urate, along with many PAMPs, including microbial toxins, inflammasome activation has proven much more complex than originally thought. Reports suggest that several stimuli do not promote direct activation of the NLRP3 inflammasome through binding to the LRR, but instead prepare the inflammasome for activation if further stimulated. For example, Bauernfeind et al. (2009) demonstrated that NOD2 stimulator, muramyldipeptide, did not provoke direct activation of NLRP3 inflammasome through caspase-1 cleavage and IL-1β release, but instead primed the NLRP3 inflammasome for subsequent activation via a second stimulus (Bauernfeind et al., 2009). An additional study performed by Harder et al. (2009) examined the effects of Streptococcus pyogenes infection on bone marrow-derived macrophages
(BMDMs) and found that NF-κB activation was crucial for subsequent caspase-1 activation.

During *S. pyogenes* infection macrophages deficient in TLR adaptors MyD88 and TRIF demonstrated impaired induction of pro-IL-1β and subsequent secretion of IL-1β (Harder *et al*., 2009). These studies, among others, suggest a more complex mechanism of action for the NLRP3 inflammasome (Bauernfeind *et al*., 2009) (Harder *et al*., 2009).

From these and other investigations, a two-signal model of inflammasome activation has been proposed in macrophages: the first signal (or priming signal) involves microbial or endogenous molecules triggering NF-κB signalling pathway to induce de-novo synthesis of pro-IL-1β and NLRP3 and the second signal (or activating signal) results in inflammasome formation, caspase-1 activation and subsequent IL-1β/IL-18 processing and release. A diagram depicting the signal involved in these two events is depicted below in Image 1.0.
1.4.1 Signal One: Inflammasome Priming

In order to better understand the priming signal required for inflammasome activation in macrophages, Schroder et al. (2012) exposed wild-type BMDMs to the NLRP3 agonists, ATP and nigericin, with or without bacterial LPS pre-treatment (Schroder et al., 2012). Increased caspase-1 cleavage was observed with the combination of LPS and NLRP3 agonists compared to NLRP3 agonists alone, an effect associated with NLRP3 gene transcription; overall, this suggests that priming is necessary for optimal inflammasome output and positively regulates the transcription of the NLRP3 gene. Additionally, this study noted that the increased caspase-1 cleavage with the combination of LPS and NLRP3 agonists occurred even at the two-hour time point, suggesting that NLRP3 inflammasome priming may be through an LPS signalling pathway, which then triggers de novo transcription of NLRP3 and pro-IL-1β (Schroder et al., 2012). Additional reports support these findings; macrophages show little activation of inflammasome with NLRP3 agonists alone, however, pre-treatment with microbial or endogenous ligands (including TLR ligands, tumor necrosis factor, or IL-β) binding to a PRR or cytokine receptor induces NLRP3 transcription and expression in through NF-κB-mediated signaling events (Schroder et al., 2012) (Juliana et al., 2012).

To elucidate the mechanism of action surrounding LPS stimulation of NLRP3 expression, Juliana et al. (2012) demonstrated that a lengthy pre-treatment of LPS was not required for NLRP3 expression, as even ten minutes was sufficient to evoke NLRP3 priming (Juliana et al., 2012). In addition, their findings suggest that in BMDMs, signaling via the TLR4 through MyD88 can quickly prime NLRP3, and prepare the inflammasome for assembly should a second stimulus be present. Furthermore, murine macrophages deficient in several signaling molecules of the NF-κB pathway including IL-1 receptor-associated kinases (IRAK1), IRAK4, and adapter
MyD88 confirm that the NF-κB signaling pathway is highly involved in the role of priming in NLRP3 activation (Juliana et al., 2012) (Fernandes-Alnemri et al., 2013) (Bauernfeind et al., 2009).

1.4.2 Signal Two: Inflammasome Activation

A number of structurally distinct entities can trigger activation of the NLRP3 inflammasome (Zhou et al., 2011) (He et al., 2016). Given the broad range of NLRP3 agonists, it is unlikely that the NLRP3 protein is directly binding to these agents, instead it is thought that a common cellular signal triggers NLRP3 inflammasome assembly and resulting activation. Several cellular mechanisms are associated with NLRP3 inflammasome activation, including reactive oxygen species (ROS) production, intracellular calcium increases, and ATP release and associated potassium (K⁺) efflux (Zhou et al., 2011) (He et al., 2016).

1.4.2.1 – ROS production and inflammasome activation

Reactive oxygen species (ROS), defined as a group of small, reactive signalling molecules endogenous to the host, includes such molecules as hydrogen peroxide, ozone, and superoxide among other free radicals (van Bruggen et al., 2010) (He et al., 2016). In spite of numerous investigations over decades, the relationship between ROS production and inflammasome activation has come under scrutiny due to the complex and puzzling nature of ROS biochemistry and biology combined with its involvement in several unique cellular pathways. Consensus between various studies can be found in the necessity of future experimentation to solidify the true nature of ROS production triggered NLRP3 activation (van Bruggen et al., 2010).

Initially observed in the macrophage-like THP-1 cells and later in BMDMs, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase related ROS production was shown to
stimulate NLRP3 inflammasome activation (Dostert et al., 2008). In these early studies, an NADPH oxidase inhibitor, diphenylene iodonium (DPI), could inhibit NLRP3 inflammasome activation triggered by MSU and ATP. This inhibition was not noted with the addition of mitochondrial complex inhibitors, suggesting that NADPH oxidase induces ROS production to trigger inflammasome activity (Dostert et al., 2008).

Other reports questioned the reliance of ROS production-related inflammasome activation on NADPH oxidase activity. Using human peripheral blood mononuclear cells lacking NADPH oxidase activity van Bruggen et al. (2010) found that the inflammasome activation could occur without NADPH oxidase, suggesting that mitochondrial-derived ROS are sufficient for NLRP3 inflammasome activation, in addition to the NADPH oxidase activity noted by Dostert and others (2008) (van Bruggen et al., 2010) (Dostert et al., 2008).

To further investigate this, Zhou et al. (2011), induced ROS production within the mitochondria of THP-1 cells by inhibiting respiratory chain enzymes, thereby inducing superoxide production (Zhou et al., 2011). As a major source of ROS within the cell stems from the mitochondria, several unique stressors, including hypoxia and membrane damage can stimulate mitochondrial ROS production. Findings from this study suggest a positive relationship between mitochondrial ROS production and IL-1β release from THP-1 macrophage (Zhou et al., 2011). Furthermore, as ROS have limited life span and restricted signalling outreach within the cell, it is suggested that NLRP3 inflammasome may require close proximity in order to be stimulated by mitochondrial ROS. Overall, this study implicates mitochondrial perturbation and resulting ROS production in NLRP3 inflammasome activation (Zhou et al., 2011) (Yuan et al., 2016).
Previous investigations have implicated ROS as an upstream activator of the NLRP3 inflammasome, and a study by Zhou and others (2010) furthered this notion with the discovery of a redox-sensitive factor of NLRP3 inflammasome activation: thioredoxin (TRX)-interacting protein (TXNIP). Using THP-1 cells and BMDMs, they found that ROS generating activators of the NLRP3 inflammasome (including MSU) triggered TXNIP to associate with NLRP3 inflammasome, initiating its activation. Overall, this study posited that, in resting state, TRX remains bound to TXNIP, preventing translocation. After an increase in ROS, TXNIP then releases TRX which causes TXNIP to interact with NLRP3 and TRX to initiate NLRP3 inflammasome activation. Ultimately, data from this study suggest that TXNIP-NLRP3 interactions are necessary for inflammasome activation in response to oxidative stress (Zhou et al., 2010). As TXNIP deficiency has been implicated in elevated insulin sensitivity, the work by Zhou and others (2010) suggests that TXNIP dysregulation may also stimulate the NLRP3 inflammasome causing inflammation and a metabolic dysfunction as seen in type 2 diabetes (Bodnar et al., 2002) (Zhou et al., 2010).

The exact role of ROS production and inflammasome activation has yet to be conclusively elucidated despite intensive investigations (van Bruggen et al., 2010) (Zhou et al., 2010) (He et al., 2016). What is known is that a variety of stimuli can induce ROS production triggering NLRP3 inflammasome activation, including NADPH oxidase related ROS production, mitochondrial disruption-related ROS, and ROS triggering TXNIP to bind to NLRP3, inducing its activation (Dostert et al., 2008) (Zhou et al., 2010) (Zhou et al., 2011). However, further inquiry is crucial to obtain a more well-rounded perspective of ROS production and its link to NLRP3 inflammasome activation.
1.4.2.2 – Intracellular $\text{Ca}^{2+}$ and inflammasome activation

An alternate pathway implicated in inflammasome activation is calcium signalling and release of calcium from intracellular stores (Feldmeyer et al., 2007) (Murakami et al., 2010). Release and mobilization of calcium triggers several unique processes within the cell, including cellular metabolism, proliferation, transcription, and in some cases, cell death. Influx of calcium from extracellular space and intracellular store from the endoplasmic reticulum (ER) can trigger rapid increase of calcium within the cytoplasm and mobilization of calcium within the cell (Katnelson et al., 2015). One such study performed by Feldmeyer et al. (2007) assessed the relationship between intracellular free calcium and NLRP3 inflammasome activation within human keratinocytes which constitutively express inflammasome substrates pro-1β, and -18 (Feldmeyer et al., 2007). They found that ultraviolet B (UVB) irradiation activated the NLRP3 inflammasome through increased intracellular calcium, as a cell permeable calcium chelator, BAPTA-AM, reduced IL-1β secretion after UVB irradiation in a concentration manner. Overall, this study demonstrated that the inflammasome-dependent activation and secretion of pro-IL-1β is inhibited with intracellular calcium chelators and supports other studies suggesting that intracellular calcium may be a general prerequisite to NLRP3 activation (Chu et al., 2009) (Feldmeyer et al., 2007). To better understand the nature of intracellular calcium increases, Murakami et al (2012) examined the role of calcium mobilization using LPS-primed BMDMs; findings suggest that the addition of ATP, nigericin, and monosodium uric acid crystals (MSU) stimulates mobilization of calcium to trigger NLRP3 inflammasome activation (Murakami et al., 2010). Moreover, this investigation demonstrated that calcium mobilization stimulated mitochondrial damage, which may not be surprising given that the mitochondria is involved in calcium signalling, as the mitochondria takes up calcium from the endoplasmic reticulum or
from the extracellular space, combined with the previous findings that damage to the mitochondria has been demonstrated to induce inflammasome activation through ROS production (Murakami et al., 2012). However, the reliance of inflammasome activation on increases in intracellular calcium is under debate – a study conducted by Katnelson et al. (2015) suggests that activation of the NLRP3 inflammasome is independent of calcium mobilization or calcium signalling within the cell (Katnelson et al., 2015). Their findings implicated potassium (K+) efflux as the key inflammasome activator, where calcium seemed to play a minor role, if any. But beyond this, they also noted an inhibition of inflammasome activation via calcium chelator, BAPTA; surprisingly, this inhibition was independent of its inhibitory effects of calcium signalling (Katnelson et al., 2015). Therefore, more investigation is crucial to understand the true role of calcium signalling in NLRP3 activation.

1.4.2.3 – K+ efflux and inflammasome activation

Potassium efflux has also been implicated in NLRP3 inflammasome activation, as LPS-stimulated mouse macrophages exhibit increased caspase-1 cleavage and IL-1β release with the addition of K+ ionophores (Perregaux et al., 1994). Potassium has also been shown to be released in response to ATP, nigericin, and other NLRP3 stimuli. These studies concluded that a decrease in intracellular K+ concentration due to its efflux into the extracellular space triggers NLRP3 inflammasome activation through triggering IL-1β release (Munoz-Planillo et al., 2013) (Petrilli et al., 2007). Petrelli and others (2007) examined the dependence of NLRP3 inflammasome activation on K+ efflux and reported that MDP-induced NLRP3 inflammasome activation was attenuated by high extracellular potassium levels (Petrilli et al., 2007). To add to this, other studies have shown that glyburide, a K+ efflux inhibitor could attenuate NLRP3 inflammasome. As a common treatment for type 2 diabetes, glyburide has been shown to inhibit ATP-induced IL-1β secretion, caspase-1 cleavage, and cell death in macrophages (Riddle, 2003) (Lamkanfi et
Glyburide has been shown to act through inhibition of ATP-sensitive K+ channels in pancreatic β cells, and this data combined with glyburide inhibition of NLRP3 inflammasome suggests that glyburide acts through blocking K+ efflux (Ashcroft, 2005) (Lamkanfi et al., 2009) (Laliberte et al., 1999). Together, these studies implicate potassium efflux upstream of and leading to NLRP3 inflammasome activation.

1.4.2.4 – Extracellular ATP and inflammasome activation

ATP signaling has also been implicated in NLRP3 inflammasome activation. Several different cellular triggers stimulate ATP release, which can then bind to and activate purinergic receptors on the cell surface to induce autocrine or paracrine activation (Lazarowski et al., 2003). The proposed mechanism of action of ATP on NLRP3 inflammasome activation is as follows: ATP is released through the gating of a pannexin-1 channel; it then interacts with purinergic receptors, unless degraded to adenosine, triggering K+ efflux and subsequent inflammasome activation. Extracellular ATP can then interact with several purinergic receptors, P2X or P2Y receptors, among others (Praetorius and Leipziger, 2009) (Iyer et al., 2009).

A study by Sutterwala et al (2006) examined the role of NLRP3 in ATP release using LPS-primed BMDMs (Sutterwala et al., 2006). Like previous findings, they demonstrated that NLRP3 and ASC were essential for caspase-1 activation in macrophages. However, they also found that NLRP3 is crucial for ATP-induced activation of caspase-1, and for the efficient secretion of IL-1β and IL-18 (Sutterwala et al., 2006). Others have reported that P2X7 receptor knockout mice fail to respond to extracellular ATP, whereas their wild-type counterparts release IL-1β in response to ATP stimulation (Solle et al., 2001). Together, these data suggest that ATP release triggers NLRP3 inflammasome activation through activation of the P2X7 receptor (Solle et al., 2001) (Sutterwala et al., 2006). However, others have reported contrasting findings. One
such recent study attempted to elucidate the underlying mechanisms in ATP-induced NLRP3 inflammasome activation (Sadatomi et al., 2017). In this report, the authors found that ATP and nigericin, all exerted unique mitochondrial membrane potential and morphology effects within BMDMs. Extracellular ATP induced the loss of mitochondrial membrane potential and mitochondrial fragmentation in a different manner than other NLRP3 agonists, suggesting that mitochondrial perturbation may play a role in ATP-induced activation of the NLRP3 inflammasome (Sadatomi et al., 2017) (Ichinohe et al., 2013).

To gain a better understanding of ATP release related NLRP3 inflammasome activation, one study tested the ability of pannexin channels to allow for passage of ATP as measured through luminometry, to elucidate whether ATP release could be mediated via this channel to trigger paracrine and autocrine binding to the P2X7 receptor (Bao et al., 2004). Their findings suggest that cell types expressing pannexin-1 channels do display ATP release when conditions are favourable. To add to this, an additional study investigated the mechanism of pannexin-1 channel activation and the dependence of pannexin-1 channel of NLRP3 inflammasome activation (Pelegrin and Surprenant, 2006). Their findings suggest that the pannexin-1 channel is a prerequisite for IL-1β release due to P2X7 receptor activation (Bao et al., 2004) (Pelegrin and Surprenant, 2006).

Overall, a channel model has been proposed to explain ATP release induced NLRP3 inflammasome activation; this is thought to involve, firstly, ATP release from pannexin-1 channel (Pelegrin and Surprenant, 2006). Following this, extracellular ATP then binds in an autocrine and paracrine fashion to the P2X7 receptor, triggering the efflux of intracellular potassium ions, thereby initiating NLRP3 activation (Lazarowski et al., 2003) (Bao et al., 2004) (Petrelli et al., 2007) (Pelegrin and Surprenant, 2006).
In summary, while numerous investigations have been performed to better understand the nature of inflammasome activation, one single mechanism has yet to be discovered (He et al., 2016). What is generally accepted is the two-signal model of inflammasome activation, with microbial or endogenous stimuli activating TLRs, IL-1Rs, or TNFR to promote downstream signalling through the NF-κB pathway to trigger de novo transcription of NLRP3 and pro-IL-1β (Bauernfeind et al., 2009) (He et al., 2016). However, current data suggest a role for ROS production, ATP release, potassium efflux, and potentially intracellularly calcium increases in stimulating NLRP3 activation (Schroder et al., 2016) (Munoz-Planillo et al., 2013) (Sadatomi et al., 2017) (Katnelson et al., 2015).

1.5 The Pregnane X Receptor (PXR)

As the role of various nuclear receptors in manipulating the immune response has emerged, certain nuclear receptors have been implicated in the progression of inflammatory diseases (Fraser et al., 2012). Growing evidence in both human and murine models reveals that numerous adopted nuclear receptors, including the PXR, play defensive roles in multiple diseases by modulating inflammatory responses (Fraser et al., 2012) (Zhou et al., 2006).

First identified as a transcriptional regulator of hepatic cytochrome P450 (CYP) 3A4, a crucial enzyme of the CYP family which is responsible for metabolizing over 50% of clinically used drugs and a corresponding quantity of xenobiotic materials, the PXR is a ligand-activated nuclear receptor encoded by NR1I2 (Fraser et al., 2012). Expressed at very high levels in the liver and small intestine, the PXR is activated through a myriad of diverse ligands both endobiotic and xenobiotic and once activated translocates from the cytoplasm to the nucleus, where it binds to a specific DNA response element as a heterodimer with the retinoid X receptor (RXR) (Lehmann et al., 1998). The PXR regulates xenobiotic metabolism via triggering oxidation (phase I genes), conjugation (phase II genes), and transport (phase III genes) of
xenobiotics in order to stimulate drug clearance from both the liver and the gastrointestinal tract (Lehmann et al., 1998) (Fraser et al., 2012), but is also involved in transcription of genes involved in metabolism and transport of endogenous molecules (Gu, 2006). Essentially, the PXR regulates a large and varied quantity of genes designed to accelerate elimination of toxic by-products derived from metabolites of exogenous substances. To date, known activation effects include increasing glucose metabolism, lipid metabolism, bile acid detoxification, drug metabolism, and inhibiting inflammatory response (Chai et al., 2013). A detailed diagram depicting the mechanism of action for the PXR can be found in Image 2.0 (Venkatesh et al., 2014).

![Image 2.0: PXR mechanism of action (Adapted from: Venkatesh et al., 2014)]
Interestingly, a recent investigation by Dash et al. (2017) explored the heterodimerization of the PXR and RXR via green fluorescent protein (GFP) or red fluorescent protein (RFP)-tagging both receptors using a HEK-293T cell line (Dash et al., 2017). From this, visualization of the intermolecular interactions between RXR-PXR occurred within the cytoplasm (instead of the classical mechanism of action previous described in the literature, nuclear heterodimerization of RXR-PXR), and data demonstrated that once RXR-PXR heterodimer occurs, translocation of the heterodimer into the nucleus was mainly driven by the RXR. Overall, this investigation demonstrates that prior to nuclear translocation, the two nuclear receptors may dimerize within the cytoplasm, triggering translocation into the nucleus and stimulating gene transcription. This finding indicates that further investigations are crucial to better understand the true nature of the PXR mechanism of action and suggests a potential role of the PXR within the cytoplasm, potentially separate from gene transcription (Dash et al., 2017).

1.6 Relationship between PXR and innate immunity

Numerous studies posit that the PXR can regulate a variety of processes related to innate immunity within the GI tract; more specifically, several investigations implicate PXR activation linked to NF-κB suppression (Cheng et al., 2012) (Terc et al., 2014) (Fraser et al., 2012) (Gu, 2006). As a major regulator of inflammation involved in both innate and adaptive immunity, NF-κB regulates the expression of a wide array of genes, including various cytokines, chemokines, and adhesion proteins. Once a ligand binds to its respective receptor, NF-κB activation leads to downstream transcription of pro-inflammatory genes. When inactive in a resting state, NF-κB remains in the cytoplasm due to IκB shielding its nuclear localization sequence. However, once active via IκB phosphorylation triggered by IκB ubiquitin-mediated
degradation, NF-κB then translocates to the nucleus where it stimulates transcription of target genes. A diagram of NF-κB mechanism of action is described in Image 3.0.

In addition to regulating metabolic function, some have reported that the PXR can regulate inflammation by interacting with NF-κB. One such study investigated the relationship between PXR activation and NF-κB activity. Zhou et al. (2006) reported that PXR activation inhibited expression of multiple NF-κB target genes, including TNF-α, IκBα, and IL-2, in human liver sample and primary hepatocytes (Zhou et al., 2006). Furthermore, the mouse PXR agonist, PCN, inhibited TNF-α–induced NF-κB target gene expression in wild-type, but not PXR−/− mouse primary hepatocytes, highlighting the role of the PXR as a negative regulator of inflammation. Additionally, histological data within the jejunum also displayed increased inflammation in PXR knockout mice compared to wild-type (Zhou et al., 2006). Altogether, data from this report implicates PXR activation in inhibition of NF-κB cell signalling pathway. Interestingly, reciprocal interactions exist between the PXR and NF-κB, as NF-κB activation inhibits PXR signalling of target gene CYP3A4 through the p65 subunit of NF-κB (Zhou et al., 2006).

Others have reported similar findings supporting the anti-inflammatory role of the PXR. Mencarelli et al. (2011) reported that TLR4-induced NF-κB activation in intestinal epithelial cells (IECs) was attenuated by stimulation of the PXR with rifaximin (Mencarelli et al., 2011). They showed that LPS-triggered increases in TNF-α, IL-1, and other NF-κB target genes were attenuated with the addition of rifaximin; effects that were not observed in PXR-deficient cells. This study also posited that the PXR-dependent inhibition of NF-κB activity occurred through the association between NF-κB p65 subunit. These findings were consistent in colon biopsies, where rifaximin attenuated NF-κB activity triggered by LPS (Mencarelli et al., 2011).
To better understand the reciprocal nature of PXR activation and NF-κB cell signalling pathways, Gu and colleagues (2006) used primary human hepatocyte culture to explore NF-κB activation and suppression of PXR target gene, CYP3A4. Other have reported that suppression of CYP3A4 expression occurs during sepsis due to inflammatory mediator secretion (Gu et al., 2006) (Renton, 2004). Gu et al. (2006) demonstrated that LPS and TNF-α stimulation of primary hepatocytes decreased PXR and CYP3A4 mRNA levels. This decrease was found to be NF-κB-dependent, as inhibition of NF-κB alleviated the suppression of CYP3A4 triggered by LPS and TNF-α. As others have reported, NF-κB subunit p65 is responsible for PXR inhibition, as NF-κB activation stimulates p65 translocation into the nucleus, disrupting the heterodimerization of PXR-RXR which then suppresses cyp3a4 expression as seen in this study (Gu et al., 2006). All together, these findings suggest a reciprocal relationship between the PXR activation and NF-κB cell signalling activity, and suggests a role for the PXR in modulating the innate immune system within the GI tract (Zhou et al., 2006) (Mencarelli et al., 2011).

1.7 Relationship between PXR and GI inflammation

Previous investigations exploring PXR gene expression suggest that dysregulation of PXR transcription and consequential down-regulation of xenobiotic metabolism within the GI tract is associated with IBD development (Langmann et al., 2004) (Dring et al., 2006). Profiling of inflamed colon tissue biopsies from IBD patients revealed a significant decrease in pxr gene expression, suggesting that the PXR may be involved in IBD pathogenesis (Langmann et al., 2004) (Dring et al., 2006). More specifically, several SNPs in PXR have been linked to decreased PXR activity in IBD patient samples (Dring et al., 2006). Because of this, several investigations have explored the role of PXR as a therapeutic target to inflammation associated with IBD (Shah et al., 2007) (Cheng et al., 2012). One such study performed by Shah et al
(2007) examined the protective role of PXR against a model of experimental colitis, (dextran sulfate sodium (DSS)) within a murine model (Shah et al., 2007). Findings revealed a high expression of PXR within the murine colon, and activation of PXR decreased susceptibility to DSS colitis, as the rodent specific PXR agonist, PCN, was shown to reduce intestinal inflammation and mucosal damage. The protective nature of PXR demonstrated in this study is suggested to be due to NF-κB inhibition, as PCN treatment suppressed NF-κB target genes within the murine colon (Shah et al., 2007). Cheng et al. (2012) examined the role of two PXR agonists and their effects on DSS and trinitrobenzene sulfonic acid (TNBS) induced colitis within a PXR−/− mouse and humanized PXR murine model (Cheng et al., 2012). They reported that pre-administration of rifaximin (a selective human PXR agonist) induced activation of hPXR, resulting in increased survival and recovery following DSS challenge, an effect not observed in wild-type or PXR−/− mice. This effect was thought to be due to inhibition of the NF-κB pathway inhibition, as rifaximin attenuated expression of NF-κB target genes (Cheng et al., 2012).

To gain a more robust understanding of PXR modulation of innate immune activation factors, Venkatesh and others (2014) explored the addition of PXR agonists and their effects on innate immune receptors (Venkatesh et al., 2014). Utilizing mice jejunum villi enterocytes within PXR−/− and wild-type mice, Venkatesh and others (2014) discovered an increase in the expression of several TLR genes within the PXR−/− model compared to control via real-time qPCR. Broadly, this investigation found a significant downregulation in anti-inflammatory mRNAs and, concurrently, an increase in pro-inflammatory cytokine mRNAs within the murine PXR−/− enterocyte model compared to the wild-type murine enterocytes (Venkatesh et al., 2014).
Overall, consensus between multiple investigations can be found in the identity of PXR as an innate immune modulator within the GI tract (Wang et al., 2014) (Venkatesh et al., 2014).

Altogether, these studies emphasize the significance of nuclear receptors in maintaining the immune system and performing the restorative functions crucial to proper gut health; therefore, the role of nuclear receptors located within the GI tract should be explored in order to expand knowledge of the microbiota and corresponding receptors present within the GI tract, and possibly bring to light novel treatments for prolonged gut inflammation.

Image 3.0 NF-κB mechanism of action (adapted from: http://www.stat.rice.edu/~siefert/Research/NfKB.html)
1.8 PXR and the NLRP3 Inflammasome

While little is known about the role of the PXR in the regulation of the NLRP3 inflammasome in innate immune cells, one report implicated PXR activation in stimulating the expression of several PRR and innate immune genes, including the NLRP3, pro-caspase-1 and pro-IL-1β (Wang et al., 2014). Utilizing a human umbilical vein endothelial cell (HUVEC) model, Wang and others (2014) exposed HUVEC cells to two structural unique PXR agonists (Rifampicin and SR12813) for 24 hours at varying concentrations. Results suggest that exposing endothelial cells to PXR agonists triggered a concentration dependent increase in NLRP3, cleaved caspase-1 and active IL-1β, as seen through western blot (Wang et al., 2014). Using a PXR knockdown, these concentration dependent increases in NLRP3, cleaved caspase-1 and active IL-1β were attenuated, suggesting that these effects were PXR dependent. These data suggest that stimulation of the PXR can trigger NLRP3 inflammasome activation in a concentration dependent manner, however the mechanism involved in this response has yet to be characterized (Wang et al., 2014).

1.9 Rationale and Summary

The limited findings concerning the relationship between PXR activation and NLRP3 inflammasome function, combined with the enigma surrounding the role of the PXR within the innate immune system, requires more attention to properly address immunity within the intestine, both within the healthy and disease GI tract. Activation of the NLRP3 inflammasome via the PXR has been demonstrated, with one report suggesting that the PXR regulates the NLRP3 inflammasome within vascular endothelial cells; however, this has not been assessed in the macrophage - a cell that is highly involved in innate immunity and mucosal homeostasis (Wang et al., 2014). Therefore, in order to extend to our understanding of how nuclear receptors affect
innate immunity, this thesis will examine the regulation of the NLRP3 inflammasome by the PXR, and the resulting mechanism of action will be assessed within both the human and murine macrophage.

Monocytes and macrophages are the major cells expressing the inflammasome genes and key producers of IL-1β release (Lopez-Castejon and Brough, 2011). As a result, many studies have focused on IL-1β production within macrophages (Lopez-Castejon and Brough, 2011) (Awad et al., 2017). Several inflammatory disorders are associated with increased IL-1β secretion and, as macrophages are key producers of IL-1β, investigations into IL-1β release is crucial to better understand the mechanisms of IL-1β secretion (Inohara and Nuñez, 2003). The rationale behind choosing macrophages to study PXR and NLRP3 inflammasome is two-fold. Firstly, macrophages are key releasers of IL-1β release and several disorders are associated with dysfunctional IL-1β secretion and so investigation into the mechanisms of IL-1β release may better elucidate the mechanism of inflammasome activation and IL-1β secretion. Secondly, human and mouse peritoneal macrophages have been shown to express the PXR, yet the PXR’s function within the macrophage has not been explored (Wallace et al., 2010) (Siest et al., 2009) (Dubrac et al., 2010).

1.10 Overarching Objectives and Hypothesis

Data generated during this thesis will enhance our understanding of the role of PXR activation and its ability to modulate the NLRP3 inflammasome within macrophages. We hypothesize that the PXR will modulate NLRP3 inflammasome function in macrophages. To test this hypothesis, I will address the following aims:
1. To evaluate the relationship between PXR activation and the expression of NLRP3, IL-1β release, and caspase-1 cleavage in PMA-differentiated THP-1 macrophages and murine macrophages

2. To assess whether stimulation of the PXR alters NLRP3 inflammasome activity

3. To determine the mechanism(s) by which the PXR modulates inflammasome activity
Chapter Two: Materials and Methods

2.1 Overarching Materials, Reagents, and Protocols

Murine peritoneal macrophages and human acute monocytic leukemia cell line (THP-1) (ATCC) were used throughout all experimental procedures. Our lab has confirmed that PXR is inherently expressed in THP-1 cells and murine macrophages; thus, all experiments were performed using these models.

2.1.1 Cell Lines, Culture, and Differentiation

THP-1 cells were propagated in RPMI 1640 medium supplemented with 10% FBS and 50 μM 2-mercaptoethanol. In order to differentiate from monocyte to macrophage, THP-1 cells were treated with 50ng/ml phorbol 12-myristate 13-acetate (PMA) for 24 hours as described in the protocol by Park and colleagues (2007) (Park et al., 2007).

2.1.2 Mouse Models

Murine macrophages were isolated from wild-type C57BL/6 mice (male and female; in-house bred) via peritoneal macrophage extraction. In each experiment, two to four wild-type mice were used to derive peritoneal macrophages and resulting macrophages were pooled. The Health Sciences Animal Care Committee from the University of Calgary have inspected and permitted the experimental procedures.

Peritoneal macrophages were isolated following the procedure by Hirota et al. (2011), where peritoneal macrophages were harvested from 4% thioglycollate-injected mice (BD Biosciences, San Jose, CA) (Hirota et al., 2011). This is in accordance with the procedure documented by Gonçalves and Mosser (2008), where peritoneal macrophages were harvested 48 hours after 4% thioglycollate and primed via 100ng/mL ultra-pure lipopolysaccharide (LPS) for thirty minutes prior to experimentation (Gonçalves and Mosser et al., 2008)
2.1.3 Reagents for PXR activation

hPXR agonist rifaximin, (Sigma Aldrich Canada, Oakville, Ontario, R9904) was used at dosages from 5-100µM as suggested by the literature, and was dissolved in sterile DMSO (Ma et al., 2007). As a vehicle control, identical volumes of DMSO were added. An alternate hPXR activator, SR12813 (Sigma Aldrich Canada, Oakville, Ontario, S4194), was dissolved in sterile DMSO to stock concentrations of 1-4µM to evaluate any agonist-specific interactions between the PXR and inflammasome activation (Moore et al., 2000). Rifaximin and SR12813 have been chosen due to their high selectivity to PXR; numerous sources confirm that rifaximin promotes PXR nuclear translocation and induces expression of CYP3A4 in vitro (Ma et al., 2006) (Moore et al., 2000).

mPXR agonist pregnenolone 16α-carbonitrile (PCN), (Sigma Aldrich Canada, Oakville, Ontario, P0543) was used at concentrations of 1-100µM as suggested by the literature, and was dissolved in sterile DMSO (Moore et al., 2000). As a vehicle control, identical volumes of stock solution were added.

2.1.4 Protein Extraction and Quantification

Protein samples were collected from both supernatant and cell lysates. Cell lysates were combined with 50µl sample buffer (25µl dithiothreitol (DTT) to 475µl 3X sample buffer (50 mM tris-HCl pH 6.8, 1% SDS, 0.001% phenol red, 3% glycerol and 1% β-mercaptoethanol)); supernatants were also combined with 50µl sample buffer for a total volume of 175µl. Samples were then boiled for ten minutes to denature proteins and placed in -20 freezer for storage for future use.
2.1.5 SDS-PAGE, Gel Electrophoresis

10μl of ExcelBand Pink Blue Protein Marker ladder (Stellar Scientific, Baltimore, MD, USA, #SMOB-PM2400) and 20μl of equal amounts of each protein sample were loaded into wells of a 10% acrylamide SDS-PAGE gel with running buffer (25mM Tris, 190mM glycine, 0.1% SDS). Proteins were then separated via a PowerPac™ HC Supply (Bio-Rad) set at 100 V for 60 to 120 minutes depending on protein of interest and corresponding molecular weight.

2.1.6 Western Blot: Protocol and Antibodies

Following SDS-PAGE protein separation, proteins were transferred onto a Polyvinylidene difluoride (PVDF) membranes (0.2 μm pores; BioRad), using transfer buffer (20% methanol, 186 mM glycine, 25mM tris-base) and the PowerPac™ HC Supply (Bio-Rad) for one hour at 100 V.

After transfer, PVDF membrane was blocked with 5% bovine serum albumin (BSA) (Fisher Scientific, Ottawa, Ontario) or non-fat, skim milk (Compliments®, Hamilton, Ontario) in phosphate buffered saline with Tween-20 (PBS-T) for one hour (shaking), and blotted with primary antibody (at 1:1000 dilution unless otherwise directed in the methods) for one hour at room temperature or in 4°C overnight on shaker. After incubation with primary antibody, diluted in either 5% bovine serum albumin (BSA) (Fisher Scientific, Ottawa, Ontario) or non-fat, skim milk (Compliments®, Hamilton, Ontario), blots were washed with PBS-T three separate times at 15 minutes each. Following this, secondary antibody (goat anti-mouse or goat anti-rabbit) diluted at 1:5000 in 5% non-fat dry milk or 5% BSA in PBS-T for 60 minutes shaking at room temperature. Following secondary antibody, blots were again washed three separate times on shaker for fifteen minutes each and imaged. Blots were then exposed to SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Scientific, Logan,
Utah, USA) shortly before blots were imaged using a Bio-Rad ChemiDoc XRS (Bio-Rad) and band intensity quantified using ImageJ (NIH) at various exposure times. If necessary, membranes were stripped using pH stripping buffer (25mM glycine and 1% SDS), re-blocked and protocol repeated as described above.

2.1.6 Enzyme-linked immunosorbent assay (ELISA)

ELISA assays were used to quantify the levels of IL-1β in THP-1 and murine macrophage culture supernatants. Assays employed a monoclonal mouse anti-human IL-1β capture antibody and a rabbit anti-human IL-1β as a detection antibody (R&D Systems, Minneapolis, MN, USA: #DLB50 and #DY401). Assays were carried out in accordance with the manufacturer's instructions using samples diluted 1/4. The amount of IL-1β release present in supernatants was determined with reference to a standard curve constructed with each assay, and a mean and standard error were calculated. Cytokine levels were expressed as pg/mL.

2.1.7 Statistical analysis

GraphPad Prism7 (La Jolla, California, USA) was used for statistical analysis, with a P value of $\leq 0.05$ considered statistically significant. The number of reproduced experimental repeats is described in figure legends, with all analyses representing at least three independent experiments expressed as mean ± standard error of the mean (SEM). Experiments were analyzed using one-way analysis of variance (ANOVA) without repeated measures when comparing more than two groups; Tukey’s multiple comparison post-tests were run to determine significant differences when comparing several treatment groups to control and also to other treatment values.
2.2 Aim 1: To evaluate the relationship between PXR activation and expression of NLRP3, IL-1β release, and Caspase-1 in THP-1 differentiated and murine macrophages

2.2.1 THP-1 cell and murine macrophage cell preparation and PXR stimulation

To clarify the role of PXR in inflammasome activation, THP-1 cells (American Type Tissue Culture Collection) were propagated as specified above. Cells were split at a 1:5 ratio upon reaching 75-80% confluence, and all experiments were performed on cell passages 10-50. The rationale behind selecting THP-1 cells revolves around its identity as an established model that parallels the human macrophage upon differentiation via PMA (Park et al., 2007).

To examine the effect of hPXR agonists on inflammasome component upregulation, THP-1 cells were seeded at a density of $5 \times 10^5$ cells/mL using standard 24-well plates (Corning) with 50ng/mL PMA to allow for differentiation into macrophages (Ma et al., 2007). After 24-hour exposure to PMA, wells were then loaded with OptiMEM ((Thermo Scientific, Logan, Utah, USA, #51985091)) containing either hPXR agonist, Rifaximin (at 5µM and 10µM) or SR12813 (at 1µM and 4µM) with 5mM ATP (Sigma Aldrich, Oaksville, Ontario, A26209) as a positive control, and matching volumes of sterile DMSO as an experimental vehicle control. Inflammasome component regulation was then assessed at six-hour time point. Experimental data was then assessed via western blot to measure NLRP3, pro-caspase-1, and cleaved caspase-1.

Additionally, to evaluate PXR activation and NLRP3 priming and activation, peritoneal macrophages were pretreated with PCN (10µM and 100µM) for 16 hours, then macrophages were pulsed with 100ng/mL ultra-pure LPS for 30 minutes. Following this, cells were stimulated with ATP (5mM) for six hours and IL-1β release in the supernatant was measured through ELISA.
2.2.2 Immunoblotting: PXR expression, Caspase-1 cleavage and NLRP3 expression

Cleaved and total caspase-1 expression were assessed using anti-caspase-1 (Santa Cruz Biotechnology Inc., Dallas, Texas, USA, #sc-622 (now discontinued), #sc-56036, and #sc-392736) antibodies, where blots were blocked in 5% skim milk, primary caspase-1 antibody was administered at 1:200 dilution overnight in 5% skim milk and washed for 30 minutes three separate times between primary antibody and secondary antibody administration, and imaging performed as described above.

NLRP3 expression was assessed using anti-NLRP3/NALP3, mAb (Adipogen, California, USA, AG-20B-0014-C100), where NLRP3 antibodies were administered following the general protocol described above.

PXR expression was assessed using anti-PXR, mAb (Abcam, Toronto, ON, Canada, ab85451) where PXR antibodies were administered following the general protocol described above.

2.3 Aim 2: Assess whether stimulation of PXR alters NLRP3 inflammasome activity

2.3.1 Inflammasome Activation Assays

To examine the effect of hPXR agonists on NLRP3 inflammasome activation, THP-1 cells were seeded at a density of 5 x 10^5 cells/mL using standard 24-well plates (Corning) with 50ng/mL PMA to allow for differentiation into macrophages (Ma et al., 2007). After exposing cells to PMA for 24 hours, wells were then loaded with Opti-MEM reduced serum medium (Thermofisher Scientific, #31985088) containing either hPXR agonist, Rifaximin (at 5µM or 10µM) or SR12813 (at 1µM or 4µM) with 5mM ATP as a positive control, and matching volumes of sterile DMSO as an experimental vehicle control. Additionally, Rifaximin (at 5µM or 10µM) and SR12813 (at 1µM or 4µM) were evaluated with the addition of ATP. NLRP3
inflammasome activation were then assessed at six-hour time point following one-hour pre-treatment with both SR12813 (at 1µM or 4µM) and Rifaximin (at 5µM or 10µM). Experimental data was then assessed via ELISA and western blot to measure IL-1β release and pro-caspase-1 and cleaved caspase-1.

Alternately, to explore the association between PXR activation and NLRP3 inflammasome activation with a different model, murine peritoneal macrophages were also examined. Following the procedure described above, peritoneal macrophages were pulsed with 100 ng/mL ultra-pure LPS for 30 minutes, pretreated with PCN (1µM, 10µM, and 100µM) for one hour, and stimulated with 5mM ATP for six hours.

2.3.2 PXR<sup>-/-</sup> murine macrophage inflammasome activation assays

Furthermore, to explore the dependence of inflammasome activation and component upregulation on PXR activation, peritoneal macrophages were extracted from C57BL/6 wild-type and PXR<sup>-/-</sup> mice (Taconic, C57BL/6-Nrl1<sup>2m3Arte</sup>, #9100-M, #9100-F). Following the procedure described above, wild-type and PXR<sup>-/-</sup> derived macrophages were pulsed with 100ng/mL ultra-pure LPS for 30 minutes, then were treated with PCN (1µM, 10µM, and 100µM), or 5mM ATP for six hours. Following this, both supernatant and cell lysates were collected for ELISA and western blot.

2.3.3 Caspase-1 inhibition

To further evaluate the relationship between PXR activation and increased caspase-1 cleavage, 10µM Z-YVAD-FMK (Biovision, #1012, Milpitas, CA, USA) a selective, irreversible caspase-1 inhibitor, was added on PMA-differentiated THP-1 cells for 30 minutes before stimulating cells with Rifaximin (at 5µM or 10µM) or SR12813 (at 1µM or 4µM) with 5mM
ATP as a positive control for 6 hours. Supernatant and cell lysates were collected for ELISA and western blot.

2.3.4 NLRP3−/− THP-1 cells and murine macrophage inflammasome activation assays

NLRP3−/− THP-1 cells were gifted from the Muruve lab (generated by Christie Sandall). Following the same procedure as described above using wild-type THP-1 cells and NLRP3−/− THP-1 cells differentiated for twenty-four hours via PMA, Rifaximin (at 5µM or 10µM) and SR12813 (at 1µM or 4µM) were evaluated with the addition of 5mM ATP as a positive control for six hours. Experimental data was then assessed via ELISA and western blot to measure IL-1β release and pro-caspase-1 in cell lysate compared to cleaved caspase-1 in the supernatant.

To ensure that increased IL-1β release and caspase-1 cleavage noted stemmed from NLRP3 inflammasome activation, peritoneal murine macrophages were extracted from C57BL/6 wild-type and NLRP3−/− mice (gifted from the Muruve lab). Following the same procedure described above, wild-type and NLRP3−/− derived macrophages were pulsed with 100ng/mL ultra-pure LPS for 30 minutes, then treated with PCN (1µM, 10µM, and 100µM), and 5mM ATP for six hours. Following this, supernatant and cell lysates were collected for ELISA and western blot to examine murine IL-1β release and cleaved caspase-1 within supernatant over total caspase-1 within the cell lysate.

2.4 Aim 3: To assess the mechanism by which the PXR modulates inflammasome activation

To examine the mechanism by which PXR activation triggers increased IL-1β release and caspase-1 cleavage, three unique pathways stimulating NLRP3 inflammasome activation were examined: intracellular calcium increase, ROS production, and ATP release/potassium release.
To explore these mechanisms, PMA differentiated THP-1 cells and murine peritoneal macrophages were used.

2.4.1 Intracellular Calcium Assay

Intracellular calcium chelator 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM, 10 μM) (Tocris Bioscience, Minneapolis, MN #2787) was added to PMA differentiated THP-1 cells 30 minutes prior to the addition of Rifaximin (at 5μM or 10μM), SR12813 (at 1μM or 4μM) and with 5mM ATP as a positive control for 6 hours. After incubation, supernatants were harvested and frozen at −20 °C. Cell lysates were harvested in 200μl of lysis buffer (20 mM Tris HCl, 137 mM NaCl, 20 mM EDTA, 10% glycerol, 0.5% Igepal, 1 mM PMSF, and protease inhibitor mixture (1:100)) and frozen at −20 °C. Following this, supernatant and cell lysates were collected for western blot to examine murine IL-1β release and cleaved caspase-1 within supernatant over total caspase-1 within the cell lysate.

2.4.2 ROS Production Assay

100μM Diphenyleneiodonium chloride (DPI) (Sigma Aldrich, Oakville, Ontario, D2926), an NADPH oxidase inhibitor used to inhibit ROS production, was added to PMA differentiated THP-1 cells 30 minutes prior to the addition of Rifaximin (at 5μM or 10μM), SR12813 (at 1μM or 4μM) and with 5mM ATP as a positive control for 6 hours. After incubation, supernatants were harvested and frozen at −20 °C. Cell lysates were harvested in 200 μl of lysis buffer and frozen at −20 °C. Following this, supernatant and cell lysates were collected for ELISA and western blot to examine murine IL-1β release and cleaved caspase-1 within supernatant over total caspase-1 within the cell lysate.
2.4.3 Inhibiting ATP release: Apyrase Assay

Apyrase (30 units/mL) (Sigma Aldrich, Oakville, Ontario, D2926) was added to PMA differentiated THP-1 cells 30 minutes prior to the addition of Rifaximin (at 5μM or 10μM), SR12813 (at 1μM or 4μM) and with 5mM ATP as a positive control for 6 hours. After incubation, supernatants were harvested and frozen at −20 °C. Cell lysates were harvested in 200μl of lysis buffer and frozen at −20 °C. Following this, supernatant and cell lysates were collected for ELISA and western blot to examine murine IL-1β release and cleaved caspase-1 within supernatant over total caspase-1 within the cell lysate.

2.4.4 Inhibiting ATP release: oxidized ATP Assay

100μM oxidized ATP (oATP) (EMD Millipore, Etobicoke, Ontario; #505758) was added to PMA differentiated THP-1 cells 30 minutes prior to the addition of Rifaximin (at 5μM or 10μM), SR12813 (at 1μM or 4μM) and with 5mM ATP as a positive control for 6 hours. After incubation, supernatants were harvested and frozen at −20 °C. Cell lysates were harvested in 200μl of lysis buffer and frozen at −20 °C. Following this, supernatant and cell lysates were collected for ELISA and western blot to examine murine IL-1β release and cleaved caspase-1 within supernatant over total caspase-1 within the cell lysate.

2.4.5 ATP release Assay

ATP was measured using CellTiter-Glo Luminescent Cell Viability Assay (Promega, #G7570). 24 hours after plating THP-1 cells differentiated with 50ng/ml PMA (5x10^5 cells/mL in a 96 well plate) in growth-factor free medium (Optimem). Rifaximin (at 5μM or 10μM), SR12813 (at 1μM or 4μM) were treated for 15 seconds, 1, 5, 10,15, and 30 minutes and with 500ng/mL MSU, pretreated for one hour, as a positive control (Gombault et al., 2012). 50μL supernatant was then added to 50μl CellTiter-Glo® reagent in a separate 96 well plate. An ATP
standard curve was generated using ATP solutions from 1 µM to 10 nM with 100 µl of 1 µM ATP solution containing $10^{-10}$ moles ATP and absorbance readings from experimental reagent samples were fit into this curve to generate concentration of ATP. Signal intensity was read on a luminometer (Perkin-Elmer).

To measure PXR dependence, ATP release was measured in PXR$^{-/-}$ and wild-type (Taconic, C57BL/6-Nr1i2tm3Arte, #9100-M, #9100-F) murine peritoneal macrophages were harvested (5x10$^5$ cells/mL in a 96 well plate), as described above, and pulsed with 100ng/mL LPS for 30 minutes. PCN (10µM, 100µM) was then added for 15 seconds, 10 minutes, and 30 minutes, with 500ng/mL MSU, pretreated for one hour, as a positive control (Gombault et al., 2012). CellTiter-Glo reagent was added, an ATP concentration curve was again performed and, as described above, signal was read on a luminometer.
Chapter Three: Results

3.1 PXR is expressed in THP-1 cells under basal conditions

Prior to assessing the role of the PXR in modulating the NLRP3 inflammasome, we first sought to ensure the presence of the PXR within macrophages. To do this, we immunoblotted for PXR within PMA-differentiated THP-1 cells. Our immunoblots revealed that the PXR was expressed in PMA-differentiated THP-1 macrophages, findings that support previous reports of PXR expression in macrophages (Wallace et al., 2010) (Figure 3-1).

**Figure 3-1: PXR is expressed in THP-1 cells.** Levels of PXR (50kD) were detected in untreated THP-1 cell lysates by immunoblot, with beta-actin measured in cell lysate as a loading control.
3.2 PXR agonists co-treated with ATP attenuates IL-1β release compared to ATP alone in mouse macrophages before priming

Previous literature by Zhou et al. (2006) has reported that activation of PXR inhibited NF-κB cell signalling, and as NF-κB signalling plays a key role in NLRP3 inflammasome priming (signal 1), we investigated the ability of PXR activation to inhibit inflammasome activation (Zhou et al., 2006) (Fernandes-Alnemri et al., 2013). To evaluate inflammasome activation in response to pre-treatment with PXR agonists, mice were injected with 4% thioglycollate. 48 hours later, peritoneal macrophages were harvested and then pretreated with mouse PXR agonist, PCN, at 10μM and 100μM for 16 hours. Following PCN pre-treatment, macrophages were pulsed with 100ng/mL ultra-pure LPS for 30 minutes to stimulate inflammasome priming. To trigger inflammasome activation, macrophages were stimulated with 5mM ATP for six hours and IL-1β release into the supernatant was measured through ELISA.

Increased IL-1β release was noted with ATP alone, as expected, however no significant IL-1β release was noted with the pre-treatment of PCN alone at either 10μM or 100μM, as shown in Figure 3-2. Interestingly, 10μM and 100μM PCN co-treated with ATP showed a significant reduction of IL-1β release compared to ATP alone. Data suggests that pre-treatment with PCN before inflammasome priming inhibits NLRP3 inflammasome activation by ATP. However, whether this reduction occurred via PXR-related inhibition of NLRP3 inflammasome activation occurred via signal 1 (priming) or signal 2 (activation) cannot be elucidated through these experiments.
Figure 3-2: PXR activation attenuates ATP-induced IL-1β secretion from mouse peritoneal macrophages. Peritoneal macrophages were harvested 48 hours after 4% thioglycollate injection. Peritoneal macrophages were then pretreated with PCN (10μM and 100μM) for 16 hours, then macrophages were pulsed with 100ng/mL ultra-pure LPS for 30 minutes. Following this, cells were stimulated with ATP (5mM) for six hours and IL-1β release in the supernatant was measured through ELISA. **denotes p<0.01 compared to no treatment and ## denotes p<0.01 compared to positive control, ATP; one-way ANOVA with Tukey’s post-hoc test. n=4 (error bars, SEM)
3.3 PXR activation does not inhibit expression of the NLRP3 inflammasome induced by ATP in THP-1 cells

To evaluate whether PXR agonist addition moderated NLRP3 inflammasome levels after priming, NLRP3 expression levels were examined, firstly, in THP-1 cells after differentiation and priming via PMA. Rifaximin (5μM or 10μM) and SR12813 (1μM or 4μM), were administered to THP-1 cells one hour prior to ATP stimulation for six hours and NLRP3 expression was evaluated via western blot (Figure 3-3A). Data displayed no significant change in NLRP3 expression levels with the addition of PXR agonists, ATP, or co-treatment of PXR agonists and ATP compared to no treatment group, suggesting that PXR does not modulate NLRP3 expression levels after priming.

As PMA is a requirement for monocyte and macrophage differentiation but also triggers priming it is not surprising to see NLRP3 expression in cell lysate however, these immunoblots revealed that PXR agonists do not influence NLRP3 expression levels within human macrophage (Cullens et al., 2015). To further explore this, peritoneal macrophages were harvested and primed with LPS for 30 minutes before adding 1μM, 10μM or 100μM PCN for 6 hours. NLRP3 expression levels were evaluated via western blot (Figure 3-3B). Results displayed no significant change in NLRP3 expression compared to control, indicating the PXR agonists within murine macrophages do not modulate NLRP3 expression levels once primed via LPS (Figure 3-3B).
Figure 3-3: PXR activation does not influence NLRP3 expression within a THP-1 cell and peritoneal macrophage. PMA differentiated THP-1 macrophages were treated with ATP (5mM), Rifaximin (5μM or 10μM), SR12813 (1μM or 4μM) alone, or PXR agonists with ATP for six hours. (A) NLRP3 expression and beta-actin expression in THP-1 cell lysate was measured via immunoblot, one representative of four unique experiments. Peritoneal macrophages were harvested and pulsed with LPS for 30 minutes prior to treatment with PCN (1μM, 10μM or 100μM) for six hours (B) NLRP3 expression and beta-actin expression in peritoneal macrophages were measured via immunoblot, representative blot of four experiments.
3.4 PXR activation does not inhibit inflammasome activation induced by ATP in primed murine peritoneal macrophages

To evaluate whether the inhibition of IL-1β release due to PXR pre-treatment followed by ATP stimulation in Figure 3-2 was due to PXR inhibiting the priming or activation signal, the activation signal was examined. Peritoneal macrophages were harvested and then pulsed with ultra-pure LPS for 30 minutes to initiate NLRP3 priming. Following this, 10μM or 100μM PCN was pre-treated for one hour before the addition of ATP (to trigger NLRP3 inflammasome activation) for 6 hours.

Pre-treatment with PXR agonist post-priming had no effect on ATP-induced IL-1β release or caspase-1 cleavage (Figure 3-4), as no changes in IL-1β release (Figure 3-4A) or cleavage of caspase-1 (Figure 3-4B) was observed with PXR and ATP treatment compared to ATP alone. Overall, data suggest that PXR stimulation does not attenuate signal two of NLRP3 inflammasome activation induced by ATP.
Figure 3-4: PXR activation does not inhibit inflammasome activation induced by ATP in mouse macrophages. Mouse peritoneal macrophages were primed with 100ng/mL LPS, pre-treated with PCN (1μM, 10μM or 100μM) for one hour, followed by treatment with ATP (5mM) for six hours. (A) IL-1β secretion was measured by ELISA; one-way ANOVA with Tukey’s post-hoc test n=3 (error bars, SEM) (B) Secretion of processed caspase-1 (p10 subunit) was detected in cell supernatants by immunoblot, pro-caspase-1 (p35 subunit) was measured in cell lysate,
with beta-actin measured in cell lysate as a loading control; *p < 0.05 vs. negative control. **p < 0.01
3.5 PXR activation does not inhibit inflammasome activation induced by ATP in THP-1 cells

To confirm that PXR activation does not inhibit the activation of the primed NLRP3 inflammasome, we performed experiments in THP-1 cells. THP-1 cells were differentiated into primed macrophages with PMA for twenty-four hours. Following this, macrophages were pretreated for one hour with one of two structurally unique human PXR agonists (rifaximin (5μM or 10μM) or SR12813 (1μM or 4μM)) prior to ATP stimulation for six hours. Supernatants and cell lysates were examined for IL-1β release (Figure 3-5A) via ELISA, and caspase-1 cleavage over pro-caspase-1 through western blot (Figure 3-5B), as displayed in Figure 3-5.

Similar to what was found in mouse peritoneal macrophages, pre-treatment with PXR agonists does not inhibit NLRP3 inflammasome activation (Figure 3-5). Neither rifaximin, nor SR12813 pre-treatment had an effect on ATP-induced IL-1β release or caspase-1 cleavage.

Overall, data from Figure 3-4 and Figure 3-5 examined the ability of PXR activation to suppress signal 2 of the NLRP3 inflammasome mechanism of action, as both THP-1 cells and peritoneal macrophages were primed with PMA or LPS, respectively. Results display no significant change in IL-1β release (Figure 3-4A, Figure 3-5A) or caspase-1 cleavage (Figure 3-4B, Figure3-5B) with PXR agonist co-treatment with ATP (a known stimulator of NLRP3 inflammasome activation) compared to ATP alone, suggesting that the inhibition noted in Figure 3-2 may be due to PXR activation suppressing the priming signal of NLRP3 inflammasome mechanism of action, but is not due to PXR-related suppression of signal two of NLRP3 inflammasome activation.
Figure 3-5: PXR activation does not inhibit inflammasome activation induced by ATP in THP-1 macrophages. PMA differentiated THP-1 macrophages were pre-treated with PXR agonists Rifaximin (5μM, 10μM), SR12813 (1μM, 4μM) for 1 hour followed by treatment with 5mM ATP for 6 hours. (A) IL-1β secretion was measured by ELISA, n=9 (Error bars, SEM) data are expressed as one-way ANOVA with Tukey’s post-hoc test (B) Secretion of caspase-1 (p10 subunit) was detected in cell supernatants by immunoblot, with beta-actin measured in cell lysate.
as a loading control. (C) caspase-1 immunoblot quantification measured via ImageJ, n=4 (error bars, SEM) *p < 0.05 vs. control ** p<0.01, **** denotes P ≤ 0.0001
3.6 PXR activation alone stimulates inflammasome activation in primed peritoneal macrophages and THP-1 cells

While data presented in previous sections show that PXR does not negatively regulate ATP-induced activation of NLRP3 inflammasome signal 2, data obtained from control experiments revealed an unexpected finding. Our data found no significant differences in NLRP3 activation once primed due to the addition of PXR agonist compared to known activator (ATP) alone, however we inadvertently stumbled upon an interesting result when comparing the function of PXR activation alone in a primed NLRP3 inflammasome compared to NLRP3 activator, ATP. What led us to these findings were priming peritoneal macrophages with LPS for 30 minutes, followed by ATP or PCN treatment (10μM or 100μM) for six hours. Supernatant and cell lysates were collected for ELISA and western blot, as displayed in Figure 3-6 and Figure 3-4B, respectively.

Perhaps surprisingly, results displayed a significant increase in IL-1β release (Figure 3-6) and caspase-1 cleavage (Figure 3-4B) with the addition of PCN at both 10μM and 100μM alone compared to no treatment, suggesting that once the NLRP3 undergoes priming, PXR activation can trigger NLRP3 inflammasome activation, initiating the IL-1β release and caspase-1 cleavage observed.

To confirm this unexpected finding, we performed experiments in PMA-differentiated, THP-1 cells. Following differentiation, cells were treated with ATP, rifaximin (5μM or 10μM), or SR12813 (1μM or 4μM). Results from this experiment indicated a significant increase in IL-1β release (Figure 3-7A) and caspase-1 cleavage (Figure 3-7B) with both PXR agonists (rifaximin (5μM or 10μM), or SR12813 (1μM or 4μM)) compared to no treatment (Figure 3-7).
suggests that once primed, human PXR activation triggers NLRP3 inflammasome activation, as displayed through increased IL-1β release and caspase-1 cleavage.

Taken together, data from Figure 3-4B, Figure 3-6 and Figure 3-7 suggest that, once primed, the NLRP3 inflammasome can be activated in response to stimulation of the PXR.
Figure 3-6: PXR activation alone triggers NLRP3 inflammasome activation in murine macrophages. Peritoneal macrophages were harvested and primed with 100ng/mL LPS, followed by treatment with ATP (5mM) or PCN (1μM, 10μM or 100μM) for six hours. IL-1β secretion was measured via ELISA; one-way ANOVA with Tukey’s post-hoc test n=4 (error bars, SEM), ****indicates p < 0.0001 compared to no treatment control.
Figure 3-7: PXR activation alone stimulates inflammasome activation in THP-1 cells. PMA differentiated THP-1 macrophages were treated with ATP (5mM), rifaximin (5μM or 10μM), or SR12813 (1μM or 4μM) for six hours. (A) IL-1β secretion was measured by ELISA, measured by one-way ANOVA with Tukey’s post-hoc test, n=9 (Error bars, SEM) (B) Secretion of processed caspase-1 (p10 subunit) and total caspase-1 was detected in cell supernatants and lysates by immunoblot compared to pro-caspase-1 (p35 subunit) (representative of four independent experiments); ** p<0.01 compared to control, ****indicates p < 0.0001
3.7 PXR<sup>−/−</sup> peritoneal macrophages do not stimulate inflammasome activation once primed with the addition of PXR agonist, PCN

Our findings implicate PXR agonists alone in inducing activation of the primed NLRP3 inflammasome. However, to ensure that the significant IL-1β release and cleaved caspase-1 levels noted with the addition of PXR agonists were due to activation of the PXR, and not a result of off-target effects, peritoneal macrophages were harvested from wild-type and PXR<sup>−/−</sup> animals. Following collection, peritoneal macrophages were primed with 100ng/mL LPS, followed by treatment with PCN (10μM or 100μM) or ATP (5mM) for six hours. Cell lysates and supernatants were then collected to examine IL-1β release and caspase-1 cleavage via western blot and ELISA.

Findings noted a significant increase in IL-1β release in wild-type murine macrophages treated with 10μM and 100μM PCN, which is consistent with our previous findings (Figure 3-8). However, no significant increase in IL-1β secretion was noted with 10μM and 100μM PCN treatment in PXR<sup>−/−</sup> murine macrophages compared to no treatment. Also, data displayed a significant attenuation in IL-1β release with 10μM and 100μM PCN treatment in PXR<sup>−/−</sup> murine macrophages compared to wild-type macrophages, and suggests that the increased IL-1β release and caspase-1 cleavage with PXR agonists noted in Figure 3-4B, Figure 3-6 and Figure 3-7 is dependent upon PXR activation within macrophages.
Figure 3-8: PXR<sup>-/-</sup> peritoneal macrophages do not stimulate inflammasome activation with the addition of PXR agonist, PCN. Peritoneal macrophages were primed with 100ng/mL LPS, followed by treatment with ATP (5mM) (experimental control) or PCN (10μM or 100μM) for six hours. IL-1β secretion was measured by ELISA, one-way ANOVA with Tukey’s post-hoc test, n=3 (error bars, SEM) *** p<0.001
3.8 PXR activation does not stimulate caspase-1 cleavage and IL-1β release in NLRP3⁻/⁻

THP-1 cells, NLRP3⁻/⁻ peritoneal macrophages, and with caspase-1 inhibition

To determine whether these effects seen with PXR activation were initiated via NLRP3 inflammasome activation, inflammasome activation was examined in NLRP3⁻/⁻ THP-1 cells and NLRP3⁻/⁻ peritoneal macrophages treated with PXR agonists. To do this, PMA differentiated wild-type and NLRP3⁻/⁻ THP-1 cells were treated with ATP, rifaximin (5μM or 10μM), or SR12813 (1μM or 4μM). To examine the dependence of NLRP3 in murine macrophages, NLRP3⁻/⁻ and wild-type peritoneal macrophages were primed with 100ng/mL LPS, followed by treatment with ATP (5mM) or PCN (10μM or 100μM) for six hours. Cleavage of caspase-1 and IL-1β secretion was measured through western blot and ELISA.

Increased IL-1β release (Figure 3-9A) and caspase-1 cleavage (Figure 3-9B) was noted in wild-type THP-1 cells treated with ATP, as expected, but also with rifaximin and SR12813 treatment, which is consistent with our previous findings in Figure 3-5. This increased IL-1β release and caspase-1 cleavage observed in wild-type peritoneal macrophages treated with PXR agonists was abolished in NLRP3⁻/⁻ THP-1 cells, as no significant IL-1β secretion was noted in NLRP3⁻/⁻ THP-1 cells compared to no treatment. Data displayed a significant suppression in IL-1β release and cleaved caspase-1 within NLRP3⁻/⁻ THP-1 cells treated with rifaximin and SR12813 treatment compared to identical treatment with wild-type THP-1 cells (Figure 3-9A, B). To add to this, a significant increase in caspase-1 cleavage was noted in wild-type peritoneal macrophages treated with ATP, 10μM and 100μM PCN compared to no treatment control (Figure 3-9C). In NLRP3⁻/⁻ peritoneal macrophages, no change in caspase-1 cleavage was observed in ATP or PCN treatment compared to no treatment control (Figure 3-9C).
Overall, our data suggest that PXR activation related IL-1β release and caspase-1 cleavage is dependent on the presence of the NLRP3 inflammasome, as NLRP3⁻/⁻ peritoneal macrophages and THP-1 cells abolished the IL-1β release and caspase-1 cleavage observed in wild-type macrophages when stimulated with PXR (Figure 3-9). Our findings suggest that activation of the PXR requires the NLRP3 inflammasome to evoke increased IL-1β secretion and caspase-1 cleavage.

To further ensure that PXR activation triggered increased IL-1β release and caspase-1 cleavage through NLRP3 inflammasome activation, PMA differentiated THP-1 cells were pretreated with 100μM Z-YVAD-fmk (a caspase-1 inhibitor) for one hour before the addition of hPXR agonists, rifaximin or SR12813, or ATP to Z-YVAD-fmk treatment for six hours. Following this, IL-1β secretion from THP-1 supernatant was measured via ELISA.

Addition of ATP triggered a significant increase in IL-1β secretion in THP-1 cells compared to control, as expected, and ATP co-treatment with Z-YVAD-fmk inhibited IL-1β secretion compared to ATP alone, suggesting that Z-YVAD-fmk does inhibit caspase-1 cleavage (Figure 3-10). Furthermore, this experiment once again demonstrated that PXR activation triggers a significant increase in IL-1β secretion compared to no treatment control as PXR agonists elevated IL-1β secretion; the addition of PXR agonists co-treated with Z-YVAD-fmk inhibited IL-1β secretion compared to rifaximin and SR12813 alone. Altogether, this suggests that PXR activation triggers increased IL-1β secretion and caspase-1 cleavage dependent on the activity of caspase-1 and presence of NLRP3 within a murine and human macrophage (Figure 3-9, Figure 3-10). Ultimately, these data implicate NLRP3 inflammasome activation as responsible for PXR activation induced IL-1β release and caspase-1 cleavage.
Figure 3-9: PXR activation does not stimulate inflammasome activation in NLRP3−/− THP-1 cells and peritoneal macrophages. PMA differentiated wild-type and NLRP3−/− THP-1 cells were treated with ATP (5mM), rifaximin (5μM or 10μM), or SR12813 (1μM or 4μM). NLRP3−/−
and wild-type peritoneal macrophages were primed with 50ng/mL LPS, followed by treatment with ATP (5mM) or PCN (1μM, 10μM or 100μM) for six hours (A) IL-1β secretion was measured by ELISA, measured via ANOVA with Tukey’s post-hoc test, n=4 (error bars, SEM). (B) Secretion of processed caspase-1 (p20 subunit) was detected in cell supernatants by immunoblot, pro-caspase-1 (p35 subunit) was measured in cell lysate. (C) Secretion of processed caspase-1 (p20 subunit) was detected in murine peritoneal macrophage cell supernatants by immunoblot compared to total caspase-1 (p35 subunit) (representative immunoblot of four independent experiments) ** p<0.01 **** indicates p<0.0001
Figure 3-10: PXR activation does not cause IL-1β release with the addition of caspase-1 inhibitor, zYVAD-fmk. PMA-differentiated THP-1 macrophages were pre-treated for 60 min with zYVAD-fmk (100 μM) then treated with ATP (5mM), rifaximin (5μM or 10μM), or SR12813 (1μM or 4μM). IL-1β secretion was measured by ELISA, by one-way ANOVA with Tukey’s post-hoc test. n=4 (error bars, SEM) * denotes p< 0.05   ** p<0.01 ****indicates p<0.0001
3.9 Characterizing of the NLRP3 inflammasome activation by the PXR: PXR activation stimulates increasing IL-1β release and caspase-1 cleavage with increasing concentrations of PXR agonists

To gain a more robust understanding of the concentrations at which PXR agonists activate the NLRP3 inflammasome, concentrations of rifaximin and SR12813 known to trigger PXR activation were chosen (Ma et al., 2007) (Lemaire et al., 2006). PMA differentiated THP-1 macrophages were treated with rifaximin (1μM, 5μM, 10μM or 100μM) and SR12813 (1nM, 10nM, 50nM, 100nM, 500nM, 1μM, 4μM, 10μM) for 6 hours and compared to a DMSO vehicle control. IL-1β release was quantified via ELISA and caspase-1 cleavage was assessed through western blot.

A significant increase in IL-1β secretion was observed starting at 5μM rifaximin and evident at 10μM and 100μM rifaximin concentration (Figure 3-11A). Likewise, an increase in caspase-1 cleavage was also noted at 5μM, 10μM, and 100μM rifaximin (Figure 3-11B). A significant increase in IL-1β release (Figure 3-12A) and caspase-1 cleavage (Figure-12B) compared to no treatment control was also noted starting at 10nM concentration of SR12813 and evident at known concentrations of PXR activation (4μM) (Figure 3-12). This data shows that rifaximin and SR12813 induce NLRP3 inflammasome activation in a concentration dependent manner (Figure 3-11, Figure 3-12).
Figure 3-11: Rifaximin stimulation of inflammasome activation in THP-1 cells is concentration-dependent. PMA differentiated THP-1 macrophages were treated with rifaximin (1μM, 5μM, 10μM or 100μM) for 6 hours and compared to a DMSO vehicle control. (A) IL-1β secretion was measured by ELISA, one-way ANOVA with Tukey’s post-hoc test, n=4 (error bars, SEM) (B) Secretion of processed caspase-1 (p20 subunit) was detected in cell supernatants
by immunoblot, pro-caspase-1 (p35 subunit) was measured in cell lysate (representative immunoblot of four independent experiments), ** p<0.01 ****indicates p<0.0001
**Figure 3-12: SR12813 stimulation of inflammasome activation in THP-1 cells is concentration-dependent.** PMA differentiated THP-1 macrophages were treated with SR12813 (1nM, 10nM, 50nM, 100nM, 500nM, 1μM, 4μM, 10μM) for 6 hours and compared to a DMSO vehicle control. (A, B) IL-1β secretion was measured by ELISA, by one-way ANOVA with Tukey’s post-hoc test, n=4 (error bars, SEM) (C) Secretion of processed caspase-1 (p20 subunit) was detected in cell supernatants by immunoblot, pro-caspase-1 (p35 subunit) was measured in cell lysate (representative immunoblot of four independent experiments), ** p<0.01 ****indicates p<0.0001
3.10 Characterizing the activation of the NLRP3 inflammasome by the PXR: PXR agonists stimulates inflammasome activation in primed THP-1 cells in a time-dependent manner

To understand the kinetics of the PXR’s modulation of NLRP3 inflammasome signal 2, we also performed time-course experiments. PMA-differentiated THP-1 macrophages were treated with rifaximin (10μM or 100μM) and SR12813 (4μM) for 0, 30 minutes and 3, 6 hours and compared to a DMSO vehicle control. IL-1β release was quantified via ELISA and caspase-1 cleavage assessed through western blot.

We noted a significant increase in IL-1β secretion starting at three hours and persisting at six hours after treatment with 10μM rifaximin (Figure 3-13A) and a significant elevation in IL-1β secretion evident at six hours with 100μM rifaximin compared to DMSO vehicle control (Figure 3-13B). Similar findings were noted with 4μM SR12813; a significant increase in IL-1β release (Figure 3-14A) and caspase-1 cleavage (Figure 3-14B) compared to DMSO control was noted as early as 30 minutes after SR12813 treatment and persistent at three hours and six hours after treatment (Figure 3-9). From this data it can be suggested that PXR agonists also activate the NLRP3 inflammasome in a time-dependent manner.
Figure 3-13: Rifaximin stimulates inflammasome activation in a time-dependent fashion in THP-1 cells. PMA differentiated THP-1 cells were treated with 10μM and 100μM rifaximin for
0 minutes, 30 minutes, 3 hours, and 6 hours compared to DMSO vehicle control. IL-1β secretion was measured by ELISA in response to (A) 10μM rifaximin and (B) 100μM rifaximin. n=4 (error bars, SEM); one-way ANOVA with Tukey’s post-hoc test *** p<0.001 **** indicates p < 0.0001
Figure 3-14: SR12813 (4μM) stimulates inflammasome activation in THP-1 cells at 0.5, 3 and 6 hours. PMA differentiated THP-1 cells were treated with 4μM SR12813 for 0 minutes, 30 minutes, 3 hours, and 6 hours compared to DMSO vehicle control (A) IL-1β secretion was measured by ELISA; one-way ANOVA with Tukey’s post-hoc test, n=4 (error bars, SEM (B) Secretion of processed caspase-1 (p20 subunit) was detected in cell supernatants by immunoblot, pro-caspase-1 (p35 subunit) was measured in cell lysate (representative immunoblot of four independent experiments) * indicates p < 0.05, ****indicates p < 0.0001
3.11 PXR-induced inflammasome does not require ROS production via NADPH oxidase

Our findings suggest that PXR activation triggers NLRP3 activation, dependent on both the presence of PXR and the presence and activity of NLRP3 inflammasome. Because of these findings, we turned our inquiry to the mechanism by which PXR modulated NLRP3 inflammasome activity. Previous reports implicate ROS production in initiating inflammasome activation once primed, as described in detail above. These findings provided rationale for our exploration of NADPH oxidase-related ROS production as the mechanism by which PXR induces NLRP3 activation (Dostert et al., 2008). To explore this, NADPH oxidase inhibitor, Diphenyleneiodonium chloride (DPI) was pretreated for 30 minutes on PMA-differentiated THP-1 cells, followed by treatment with ATP, PXR agonists alone, or PXR agonists co-treated with DPI for six hours. Following this, ELISA and western blot analysis were performed to determine whether the addition of DPI attenuated PXR-related increased (Figure 3-15A) IL-1β and (Figure 3-15B) cleaved caspase.

No significant change in either IL-1β secretion (Figure 3-15A) or caspase-1 cleavage (Figure 3-15B) was noted with addition of DPI with rifaximin or SR12813 compared to PXR agonists alone. However, in agreement with previous literature implicating that co-treatment with DPI inhibits ATP-induced IL-1β release, data from this experiment reveal an inhibition of our positive control, ATP, when co-treated with DPI compared to ATP alone (Figure 3-15A) (Dostert et al., 2008). Data from this experiment set submit that the DPI treatment is able to attenuate the positive control, as expected, and suggest that inhibition of NADPH oxidase does not trigger attenuate of either IL-1β secretion of caspase-1 cleavage seen with the addition of
PXR agonists alone (Figure 3-15). Together, our findings in Figure 3-15 do not implicate ROS production by NADPH oxidase in the PXR related activation of the NLRP3 inflammasome.

However, it is important to note here that only one route of ROS production was examined, as ROS production is also generated in the form of hydrogen peroxide and hydroxyl radical in addition to superoxide (van Bruggen et al., 2010). Because of this, the data collected in Figure 3-15 only suggests that inhibition of the ROS superoxide is not involved in the PXR related activation of the NLRP3 inflammasome and future avenues to explore to further elucidate the potential role of other ROS in PXR induced activation of the NLRP3 inflammasome.
Figure 3-15: PXR activation stimulating inflammasome activation is not attenuated with ROS production inhibitor, Diphenyleneiodonium chloride treatment (DPI). PMA-differentiated THP-1 macrophages were pre-treated for 30 minutes with DPI (100μM) then treated with ATP (5mM), rifaximin (5μM or 10μM), or SR12813 (1μM or 4μM). (A) IL-1β secretion was measured by ELISA, and evaluated by one-way ANOVA with Tukey’s post-hoc test, n=6 (error bars, SEM) (B) Secretion of processed caspase-1 (p20 subunit) was detected in cell supernatants by immunoblot compared to total caspase-1 (p35 subunit) (representative immunoblot of four independent experiments), * denotes p<0.05  *** p<0.001 **** p<0.0001
3.12 PXR-associated inflammasome activation is not driven by intracellular calcium

While our data suggest that PXR activation triggers NLRP3 activation, the mechanism of action by which the PXR stimulates NLRP3 inflammasome activation was not reliant on ROS production via NADPH (Figure 3-15). Therefore, we wanted to explore alternate NLRP3 inflammasome activators that may be triggered by PXR activation to evoke increased the IL-1β and cleaved caspase-1 observed. To further explore this, BAPTA-AM, a cell permeable calcium chelator was chosen due to its high selectivity to control the levels of intracellular calcium over other ions to evaluate the role of intracellular calcium in PXR-driven inflammasome activation (Ainscough et al., 2015). As described by Ainscough et al. (2015) PMA-differentiated THP-1 macrophages were pre-treated for 30 minutes with BAPTA-AM (10μM) then treated with ATP (5mM) or PXR agonists (Ainscough et al., 2015). NLRP3 inflammasome activation was examined through assessing IL-1β release and caspase-1 cleavage.

We found that the addition of BAPTA-AM with PXR agonists did not attenuate IL-1β release (Figure 3-16A) or caspase-1 cleavage (Figure 3-16B) triggered by PXR agonists rifaximin or SR12813 alone (Figure 3-16). However, in keeping with previous reports, BAPTA-AM was able to significantly inhibit ATP induced IL-1β release compared to ATP alone suggesting that BAPTA-AM was administered at working concentrations and at a timepoint to induce calcium chelation (Figure 3-16A) (Feldmeyer et al., 2007). Data from this experiment set suggest that inhibition of intracellular calcium does not attenuate either IL-1β secretion or caspase-1 cleavage noted with the addition of PXR agonists, rifaximin and SR12813, alone (Figure 3-16). Together, our findings in Figure 3-16 do not implicate intracellular calcium increase as the mechanism of action by which PXR activation initiates NLRP3 inflammasome activation.
Figure 3-16: PXR activation stimulating inflammasome activation is not attenuated with intracellular calcium chelator, BAPTA-AM treatment. PMA-differentiated THP-1 macrophages were pre-treated for 30 minutes with BAPTA-AM (10μM) then treated with ATP (5mM), rifaximin (5μM or 10μM), or SR12813 (1μM or 4μM). (A) IL-1β secretion was measured by ELISA; one-way ANOVA with Tukey’s post-hoc test n=6 (error bars, SEM) (B) Secretion of processed caspase-1 (p20 subunit) was detected in cell supernatants by immunoblot.
compared to total caspase (p35 subunit) with treatment of rifaximin and SR12813 (representative immunoblot of four independent experiments) * denotes p< 0.05  ** p<0.01. *** p<0.001 ****indicates p<0.0001
3.13 PXR-associated inflammasome activation involves extracellular ATP.

Given that PXR activation of the NLRP3 inflammasome does not involve ROS production or intracellular calcium increases, we next sought to evaluate the role of ATP in our system. ATP release through the pannexin-1 channel and subsequent binding of eATP to P2X7 channels have been shown to trigger NLRP3 inflammasome activation (Lazarowski et al., 2003) (Bao et al., 2004) (Petrelli et al., 2007) (Pelegrin and Surprenant, 2006). To evaluate this, cells were exposed to apyrase, which breaks down extracellular ATP, and challenged with PXR agonists (Iyer et al., 2009). PMA-differentiated THP-1 macrophages were pre-treated for 30 minutes with apyrase (30units/mL) then treated with 5mM ATP, rifaximin (5μM or 10μM), or SR12813 (1μM or 4μM). NLRP3 inflammasome activation was examined through IL-1β release and caspase-1 cleavage and total caspase-1.

We found a significant increase in IL-1β secretion and caspase-1 cleavage with both rifaximin (Figure 3-17A, C) and SR12813 (Figures 3-17B, D) treatment; the addition of apyrase with PXR agonists attenuated both IL-1β secretion (Figure 3-17A, B) and caspase-1 cleavage (Figure 3-17C, D). Results displayed an attenuation in IL-1β release and caspase-1 cleavage in both rifaximin and SR12813 with co-treatment of apyrase (Figure 3-17). This suggests that the mechanism by which PXR stimulates NLRP3 inflammasome activation involves the presence of ATP, as inhibition of ATP through apyrase resulted in a significant inhibition of both IL-1β release and caspase-1 cleavage.
Figure 3-17: PXR activation stimulating inflammasome activation is attenuated with ATP inhibitor, apyrase (APY). PMA-differentiated THP-1 macrophages were pre-treated for 30 minutes with apyrase (30units/mL) then treated with ATP (5mM), (A, C) rifaximin (5μM or 10μM), or (B, D) SR12813 (1μM or 4μM). (A, B) IL-1β secretion was measured by ELISA; one-way ANOVA with Tukey’s post-hoc test n=6 (error bars, SEM) (C, D) Secretion of processed caspase-1 (p20 subunit) was detected in cell supernatants by immunoblot and procaspase-1 was
measured in cell lysate (p35 subunit) (representative immunoblot of four independent experiments). *** p<0.001 ****indicates p<0.0001
3.14 PXR-associated inflammasome activation involves activation of the P2X7 receptor.

As a significant inhibition in IL-1β release and caspase-1 cleavage was noted with the addition of apyrase with PXR agonists, we next wanted to evaluate whether PXR-induced NLRP3 inflammasome activation involved the activation of the P2X7 receptor. A number of groups have reported that extracellular ATP can activate the NLPR3 inflammasome by interacting with P2X7 (Solle et al., 2001) (Zhou et al., 2011) (He et al., 2016). To evaluate whether PXR related IL-1β release and caspase-1 cleavage increases were dependent on P2X7 activation, oxidized ATP (oATP), an irreversible antagonist of P2X receptors, including P2X7, was used (Grahames et al., 1999). PMA-differentiated THP-1 macrophages were pre-treated for 30 min with oATP (100μM), then treated with ATP, rifaximin or SR12813 at various concentrations. NLRP3 inflammasome activation was examined through measuring IL-1β release and caspase-1 cleavage.

Our findings indicated an increase in IL-1β release (Figure 3-18A, B) and caspase-1 cleavage (Figure 3-18C) with PXR agonist treatment alone; however, these NLRP3 inflammasome activation markers were inhibited with the addition of oATP. Overall, oATP pre-treatment resulted in a significant inhibition in IL-1β release and caspase-1 cleavage, evoked by both the addition of rifaximin (Figure 3-18A, C) and SR12813 (Figure 3-18B, C). Data presented here suggests that PXR triggers activation of P2X7 receptor to evoke NLRP3 inflammasome activation and initiate IL-1β release and caspase-1 cleavage.
Figure 3-18: PXR activation stimulating inflammasome activation is attenuated with P2X7 receptor inhibitor, oxidized ATP. PMA-differentiated THP-1 macrophages were pre-treated for 30 min with oATP (100µM) then treated with ATP (5mM), rifaximin (5µM or 10µM), or SR12813 (1µM or 4µM). (A, B) IL-1β secretion was measured by ELISA, one-way ANOVA with Tukey’s post-hoc test n=6 (error bars, SEM). (C) Secretion of processed caspase-1 (p20 subunit) was detected in cell supernatants by immunoblot and compared to total caspase-1 (p35 subunit).
subunit) (representative immunoblot of four independent experiments), ** p<0.01. *** p<0.001
****indicates p<0.0001
3.15 PXR activation triggers ATP release in a time sensitive manner.

Given that PXR activation of the NLRP3 inflammasome was attenuated with apyrase and oATP, which suggests that PXR activation triggers ATP binding to P2X7 receptors and triggering NLRP3 inflammasome activation, we next wanted to assess whether PXR activation stimulated ATP release. PMA-differentiated THP-1 macrophages were treated with rifaximin or SR12813 at various concentrations for 15 seconds, 1 minute, 5 minutes, 10 minutes, 15 minutes and 30 minutes. As a positive control, 500ng/mL of MSU was added to THP-1 cells for an hour prior to measurements, based on the data reported by Riteau and others (2012) (Riteau et al., 2012).

We found a significant increase in ATP release with PXR agonists, 4μM SR12813 and 5μM and 10μM rifaximin, starting at the 15 second time point (Figure 3-19A). This significant increase in ATP release compared to no treatment was noted with PXR agonists at 1 minute, 5 minutes, 10 minutes, and 15 minutes (Figure 3-19B, C, D, and E). ATP release was measured until the 30-minute time-point, at which time 4μM SR12813 and 5μM rifaximin no longer stimulated a significant increase in ATP compared to control but 10μM rifaximin still triggered a significant increase (Figure 3-17F). These data suggest that PXR activation triggers ATP release shortly after treatment (15 seconds) and decreasing substantially by the 30-minute time-point. Furthermore, our data suggest that PXR activation triggers ATP release that, once released, binds to P2X7 receptor and stimulate NLRP3 inflammasome activation, thereby inducing IL-1β release and caspase-1 cleavage.
**Figure 3-19: PXR activation triggers rapid ATP release from PMA-differentiated THP-1 macrophages.** Celltiter-Glo luminescent cell viability assay was used, where PMA-differentiated THP-1 macrophages were treated with rifaximin (5μM or 10μM), or SR12813 (1μM or 4μM) for (A) 15 seconds, (B) 1 minute, (C) 5 minutes, (D) 10 minutes, (E) 15 min and (F) 30 minutes. 50μL of cell titer reagent was added to 50μL supernatant and measured via luminometer; one-way ANOVA with Tukey’s post-hoc test n=3-4 (error bars, SEM), * p<0.05 compared to no treatment control * denotes p < 0.05 , ** p<0.01 *** p<0.001 ****indicates p<0.0001
3.16 Murine PXR agonist activation triggers ATP release in a time sensitive manner and is dependent on PXR

Findings suggest that PXR activation triggers NLRP3 inflammasome activation through stimulating ATP release with macrophages. However, findings in Figure 3-17, 3-18, and 3-19 did not evaluate the dependence of ATP release on the PXR, which is crucial to determining the mechanism responsible for PXR-related NLRP3 inflammasome activation. Therefore, we assessed the release of ATP from PXR−/− macrophages compared to wild-type macrophages.

As expected, a significant increase in ATP release was noted with the addition of positive control, MSU, in both wild-type and PXR knockout macrophages. Similarly, PCN treatment, to selectively activate the mouse PXR, triggered an increase in ATP release at 15 seconds, (Figure 3-20A) 10 minutes (Figure 3-20B) with ATP release only evident with 100μM PCN at the 30-minute time-frame (Figure 3-20C). Overall, data suggest that PXR-associated NLRP3 inflammasome activation involved PXR-dependent ATP release (Figure 3-20).
Figure 3-20: mPXR agonist activation triggers ATP release in a time sensitive manner and is dependent on PXR. Celltiter-Glo luminescent cell viability assay was used, where PXR knockout and wild-type peritoneal macrophages were extracted 48 hours after 4% thioglycollate injection. Peritoneal macrophages were primed with 100ng/mL LPS for 30 minutes, then treated with positive control 500ng/mL MSU for an hour or treated with PCN (1μM, 10μM or 100μM) for (A) 15 seconds, (B) 10 minutes and (C) 30 minutes. 50μL of cell titer reagent was added to 50μL supernatant and measured via luminometer; one-way ANOVA with Tukey’s post-hoc test n=4 (error bars, SEM), ** p indicates <0.01 **** p<0.0001
Chapter Four: Discussion

The PXR has traditionally been thought of as a xenobiotic sensing nuclear receptor involved in regulating drug metabolism within the liver, small and large intestine, however studies suggest a role for the PXR in modulating the innate immune system (Lehmann et al., 1998) (Zhou et al., 2006) (Mencarelli et al., 2011) (Wang et al., 2014). Demonstrated to suppress NF-κB cell signalling within various model systems resulting in injury resolution and repair, the true role of the PXR in the context of modulating innate immune function has yet to be completely characterized (Zhou et al., 2006). Thus, the main objective of this thesis was to evaluate the role of the PXR to modulate the NLRP3 inflammasome.

In the current report, we sought to characterize the role of the PXR in modulating the function of the NLRP3 inflammasome in macrophages, a key innate immune cell that contributes to host-defense and the regulation of intestinal mucosal homeostasis (Mosser et al., 2008). Using the THP-1 cell line and mouse peritoneal macrophages, our findings suggest that PXR agonists stimulate caspase-1 activation, along with IL-1β processing and release, through activation of the NLRP3 inflammasome. Furthermore, PXR-deficient cells failed to activate caspase-1 and release IL-1β in the response to the addition of PXR agonists. Finally, we found that PXR activation stimulated ATP release, an effect that was responsible for inflammasome activation, as these responses were abolished by apyrase and P2X7 inhibition. Throughout this thesis we have demonstrated a novel role for the PXR in modulating the NLRP3 inflammasome activation within macrophages.

The innate immune system is the first line of defense against pathogens, and one mechanism by which the immune system defends against attack is via endogenous and microbial molecules binding to PRRs and inducing a receptor-specific innate immune response to eliminate
injury and maintain tissue homeostasis (Baroja-Mazo et al., 2014). Activation of these PRRs are crucial to prime inflammasome for subsequent activation, which is shown to trigger clearance of pathogens and repair of cellular damage induced by PAMPS or DAMPS (Baroja-Mazo et al., 2014) (Wang et al., 2014).

This NLRP3 inflammasome priming is a necessary first signal to prepare for NLRP3 inflammasome activation, should an alternate stimulus be present. It is now well established that NF-κB signalling plays a key role in de novo transcription of NLRP3 and pro-IL-1β observed as the result of inflammasome priming (Ogura et al., 2001) (Abbott et al., 2004) (McDonald et al., 2005). As a sensor of pathogens and their by-products, as well as endogenous molecules, NLRP3 has also been shown to be activated by ATP, ROS, intracellular calcium increases, and potassium efflux, among other stimuli. NLRP3 inflammasome activation and expression maintain immune homeostasis, and NLRP3 inflammasome dysfunction has been shown to contribute to several immunological disorders. Overall, NLRP3 inflammasome activation is complex – overexpression and suppression of inflammasome activation both have adverse consequences (Xiao et al., 2013) (Marneros et al., 2013).

Reports from the literature implicate PXR activation in the inhibition of NF-κB cell signalling, with reciprocal interactions between PXR and NF-κB cell signalling (Zhou et al., 2006) (Mencarelli et al., 2011). Reports indicate that TLR4-induced NF-κB activation in IECs is attenuated with rifaximin treatment; this was shown to be dependent on PXR (Mencarelli et al., 2011). Because of these studies, we hypothesized that the PXR might regulate the NLRP3 inflammasome, in part, by modulating NF-κB-dependent signal 1 (priming) (McDonald et al., 2005) (Mencarelli et al., 2011) (Zhou et al., 2006). In the early experiments conducted during this thesis, we found that stimulating the PXR prior to priming attenuated ATP-induced IL-1β
secretion. However, from these experiments, we could not conclude definitively whether the reduced IL-1β response was due to an inhibition of signal 1 (priming) or an effect on distal NLRP3 inflammasome activation (signal 2). Our experimental approach was to first assess whether PXR activation could directly modulate NLRP3 inflammasome activation (signal 2).

To assess NLRP3 inflammasome activation (signal 2) we used both PMA-differentiated THP-1 macrophages (which are primed) and mouse peritoneal macrophages (LPS-pulsed prior to stimulation for priming) (Daigneault et al., 2010) (Zhang et al., 2008). We hypothesized that PXR agonists were reducing ATP-induced IL-1β production through a direct inhibitory effect on NLRP3 inflammasome activation. Contrary to our hypothesis, treating macrophages with PXR prior to NLRP3 inflammasome activation (signal 2) had no inhibitory effect on caspase-1 activation or IL-1β production, suggesting that the reduction in IL-1β release observed in our earlier experiments was likely due to an inhibition of signal 1 priming, instead of a direct inhibitory effect on inflammasome activation. Interestingly, while activation of the PXR did not inhibit ATP-induced NLRP3 inflammasome activation (signal 2), exposing macrophages to PXR agonists alone initiated caspase-1 activation and IL-1β release in a time- and concentration-dependent manner, suggesting that, once primed, the NLRP3 inflammasome can be activated by activation of the PXR. These data, while surprising, and not considered in our original hypotheses, are supported, in part, by a report wherein Wang and others (2014) who found that activation of the PXR within vascular endothelial cells triggered NLRP3 inflammasome activation (Wang et al., 2014). They found that rifampicin and SR12813 triggered both priming and NLRP3 inflammasome activation, in a PXR-dependent manner (Wang et al., 2014). While the activation of the NLRP3 inflammasome by the PXR discovered in our experiments is in line with the findings by Wang and others (2014) we did not test whether PXR activation alone could
evoke priming (signal 1) in NLRP3 inflammasomes within macrophages and this could be an area to assess in future studies.

While there are currently no reports linking the PXR with the NLRP3 inflammasome in macrophages, reports indirectly support my thesis conclusions. As mentioned in previous sections, the PXR has a promiscuous ligand binding domain and can be activated by a variety of pharmaceutical compounds (Lehmann et al., 1998). Interestingly, vincristine and paclitaxel, two known PXR agonists, have been reported to trigger IL-1β release in BMDMs in an NLRP3 inflammasome-dependent manner (Wong et al., 2014) (Harmsen et al., 2009) (Xu et al., 2014). While the authors of these papers did not implicate the PXR in the responses triggered by vincristine and paclitaxel, their findings, in light of those reported in this thesis, suggest further investigation is needed to properly assess mechanism.

Throughout the course of my thesis, the data generated pointed towards a novel role of the PXR within macrophages. While there are currently no reports on the nature of PXR activation within a macrophage, the presence of the PXR has been found in other innate immune cell populations including T-lymphocytes, CD19+ B lymphocytes, and CD14+ monocytes (Schote et al., 2007) (Siest et al., 2009) (Dubrac et al., 2010). The role of PXR has been explored in T-lymphocytes. A report by Dubrac and others (2010) found expression of PXR within the activated T-lymphocyte (Dubrac et al., 2010). Their findings suggest that PXR modulates innate immune function, as PXR activation inhibited T-cell proliferation and surface marker expression (Dubrac et al., 2010). However, to our knowledge, to date, no other functional data exists regarding immune cell regulation by the PXR.
Indeed, activation of PXR in our macrophage systems with a structural diverse array of agonists triggered IL-1β production in a caspase-1- and NLRP3-dependent manner. Given the novel nature of our findings, it was imperative that we definitively implicate the PXR in these responses. As hypothesized, PCN-(rodent PXR agonist) induced NLRP3 inflammasome activation was completely lost in PXR⁻/⁻ macrophages. Furthermore, PCN-induced ATP release was absent in PXR⁻/⁻ macrophages. This suggests that the activation of caspase-1 and increased IL-1β release observed in our experiments involved some PXR-dependent event. This also supports findings by Wang et al. (2014), as they showed that a definitive link between PXR activation, caspase-1 activation and IL-1β secretion using a knockout approach (Wang et al., 2014).

While the data presented in this thesis are the first to link the PXR and NLRP3 inflammasome in macrophages, previous reports in the literature suggest that other nuclear receptors may also trigger NLRP3 inflammasome activation (Al-Daghri et al., 2014) (Tulk et al., 2015). The vitamin D receptor (VDR), has been shown to trigger NLRP3 inflammasome activation in THP-1 cells (Al-Daghri et al., 2014). Using a similar model to that employed in many of our experiments, Tulk et al. (2015) found that VDR activation triggered NLRP3 inflammasome activation and IL-1β release (Tulk et al., 2015). Interestingly, the VDR belongs to the same receptor superfamily as the PXR, and shares some similarities in intracellular signaling characteristics, suggesting further investigation into the role of nuclear receptors and inflammasome modulation might be warranted (Krasowski et al., 2011). While the findings of Tulk et al. (2015) are supportive of our results, they did not completely interrogate the mechanism(s) by which the VDR triggered NLRP3 inflammasome activation. Furthermore, the magnitude of IL-1β secretion was substantially less in their report, suggesting that different
nuclear receptors may exhibit varying degrees of NLRP3 inflammasome activation (Tulk et al., 2015).

Several mechanisms have been implicated in signal two of inflammasome activation, including ROS production, intracellular calcium increase, and ATP release. In this thesis each of these mechanisms were explored to elucidate the mechanism by which PXR activation triggers NLRP3 inflammasome activation (Schroder et al., 2016) (Munoz-Planillo et al., 2013) (Sadatomi et al., 2017) (Katnelson et al., 2015). Diphenylene iodonium (DPI), an NADPH oxidase inhibitor that suppresses ROS production, has been shown to inhibit NLRP3 activation triggered by ATP, nigericin, and silica (Bauernfeind et al., 2009) (Dostert et al., 2008). In my experiments, I found that DPI pre-treatment had no effect on the NLRP3 inflammasome activation driven by PXR stimulation, suggesting that ROS production via NADPH oxidase is not involved in our mechanism. However, it is important to note that NADPH oxidase-independent and mitochondrial ROS production have also been implicated in NLRP3 inflammasome activation (van Bruggen et al., 2010). Thus, future studies could be performed to determine whether PXR activation triggers ROS production from other sources.

To assess the role of intracellular calcium in the responses triggered by PXR activation, we used BAPTA-AM, a cell permeable calcium chelator (Chu et al., 2009). BAPTA-AM has been shown to inhibit inflammasome activation and one report stipulated that BAPTA-AM treatment on LPS-primed BMDMs resulted in suppression of inflammasome activation triggered by ATP (Murakami et al., 2010). In my experiments, BAPTA-AM had no effect on the activation of the NLRP3 inflammasome triggered by PXR agonists, suggesting that intracellular calcium increases are not involved in our system.
ATP is also implicated in triggering NLRP3 inflammasome activation. Released through a pannexin-1 channel, ATP can bind to purinergic receptors (including P2X7 receptor), triggering potassium efflux and inflammasome activation (Praetorius and Leipziger, 2009) (Iyer et al., 2009). To examine whether PXR activation triggered inflammasome activation via ATP release, we treated cells with APY to catalyse the hydrolysis of ATP to AMP (Moeckel et al., 2014). Interestingly, APY attenuated caspase-1 cleavage and IL-1β secretion in response to PXR activation, suggesting that the release of ATP could be driving NLRP3 inflammasome activation in our system. To assess this further, see sought to inhibit P2X7, the target of extracellular ATP, with the irreversible antagonist oATP (Grahames et al., 1999). As expected, blockade of P2X7 with oATP attenuated NLRP3 inflammasome activation following PXR stimulation.

Given that the data generated from my experiments pointed towards a role for extracellular ATP in the activation of the NLRP3 inflammasome by the PXR, we sought to determine whether its activation could trigger ATP release. While there are no reports linking the PXR to ATP release, bile acids, known to activate the PXR, were reported to trigger ATP release from pancreatic exocrine cells. This then can trigger purinergic signalling leading to intracellular calcium increases with the pancreatic cell lines used (Kowal et al., 2015). In my experiments, I found that PXR activation triggered ATP release in a time-dependent manner. Indeed experiments performed using PXR−/− macrophages definitively linked PXR activation to ATP release. Taken together, the data from my experiments indicate that NLRP3 inflammasome activation mediated by the PXR involved the release of ATP and subsequent P2X7 activation.

While my data have identified key steps linking the PXR to the NLRP3 inflammasome, like many studies implicating the release of ATP in NLRP3 inflammasome activation, I have yet to completely elucidate its efflux mechanism(s). It has been demonstrated ATP can be released
from cells through pannexin channels (Chekeni et al., 2010). The pannexin-1 channel is a plasma membrane ion channel that allows for transport of molecules up to approximately 1kDa (D’hondt et al., 2009). Pannexin-1 has been shown to allow for efflux of ATP, among other small molecules, and pannexin-1 mediated ATP release has been shown to trigger neutrophil and macrophage chemotaxis, as well as inflammasome activation (Adamson and Leitinger, 2014) (D’hondt et al., 2009). Under normal conditions, the pannexin-1 channel remains closed, but will open to emit small molecules and evoke paracrine and autocrine cell signals (Chekeni et al., 2010). While pannexin-1 activation is not completely understood, Chiu and others (2017) recently examined the pannexin-1 mechanism of action and determined the distal C terminus of the pannexin-1 channel yields the channel closure noted under basal conditions and restrains channel gating (Chiu et al., 2017). Rearrangement of the C-tail will then lead to increasingly larger molecules transporting through the channel (Chiu et al., 2017). Pannexin channels have been shown to engage with P2X receptors at the cell membrane; it has been demonstrated that pannexin-1 receptor is required for release of mature IL-1β and cleavage of caspase-1 induced by P2X7 receptor (Pelegrin and Surprenant, 2006). This pannexin-1 channel is key in the channel model of NLRP3 inflammasome activation within a macrophage, as described in detail above (Pelegrin and Surprenant, 2006). Our data suggests that PXR activation triggers ATP release, and suggests that this eATP subsequently binds to P2X7 receptor to evoke NLRP3 inflammasome activation; however, we have yet to characterize the mechanism of the ATP release. Future studies should focus on how PXR activation changes the characteristics of the pannexin-1 channel to facilitate ATP release.

The kinetics of PXR-dependent ATP release and NLRP3 inflammasome activation suggest a potential role of the PXR outside nuclear translocation and transcription of PXR target
genes. The defined mechanism of action for PXR activation involves sensing a ligand, translocating to the nucleus, forming a heterodimer, and triggering target gene transcription; however, the ATP release noted with the addition of PXR agonists (starting as early as 15 seconds) does not fit the time frame of transcription and translation of target genes. This suggests a potential role of the PXR outside of target gene transcription and potentially from within the cytoplasm, triggering pannexin-1 gating, ATP release and NLRP3 inflammasome activation.

Interestingly, we are not the first to discover that nuclear receptor agonists can trigger rapid responses within various organ systems, as a few steroid receptors have been documented to evoke swift responses that do not fit the time frame of gene regulation (Levin and Hammes, 2016). Results indicate that steroid receptors can localize to the plasma membrane, among other locations, and activate alternate signalling pathways divergent from their target gene transcription (Levin and Hammes, 2016) (Revankar et al., 2005). One such study evaluated the vitamin D receptor and its ability to trigger rapid responses; they proposed a VDR ensemble model that suggests that receptors can be triggered to undergo two separate types of responses: rapid responses in cytoplasm and slow responses within the nucleus. This is hypothesized to be a result of two unique ligand-binding pockets, however the true identity behind rapid responses evoked by the nuclear receptor, VDR, is not known (Norman et al., 2005) (Revankar et al., 2005) (Mizwicki et al., 2009). However, to date, little to no information regarding PXR related rapid responses has been reported. Therefore, future investigations surrounding the role of the PXR outside the defined mechanism of action is crucial to better understand the true function of PXR and other nuclear receptors within the cell.

Our findings suggest that PXR activation trigger NLRP3 inflammasome through evoking ATP release, which suggests a novel role for this nuclear receptor in evoking an innate immune
response within macrophages. Ultimately, this thesis will further the understanding of the nature of NLRP3 activation and the role of PXR within an innate immune cell which plays a key role in host defense. But beyond extending NLRP3 biology, these findings could lead to therapeutic approaches to NLRP3 dysfunction. More broadly, activation of the PXR and CYP3A4 induction has been implicated in worsening hepatotoxicity caused by acetaminophen (APAP), which, while harmless at therapeutic concentrations, can cause sterile inflammation and hepatotoxicity upon overdose (Rubartelli et al., 2013). Reports suggest that the PXR may play a role in the induction of APAP toxicity, as PXR-knockout mice exhibit lower levels of hepatic inflammation and hepatotoxicity compared to wild-type mice when treated with toxic levels of APAP (Cheng et al., 2009). Interestingly, the NLRP3 inflammasome has been implicated in inducing sterile inflammation in the liver (Szabo and Petrasek, 2015). Furthermore, one report demonstrated that triggered acetaminophen-induced hepatotoxicity and liver injury was driven by the NLRP3 inflammasome (Imaeda et al., 2009). To further support this, other have reported the involvement of P2X7 receptor in acetaminophen- induced hepatotoxicity (Hoque et al., 2012). Data from this thesis may link these two observations, suggesting that acetaminophen-induced hepatotoxicity may be due to PXR-related NLRP3 inflammasome activation. To add to this, P2X7 receptor significantly increased acetaminophen related liver necrosis, which further implicates the NLRP3 inflammasome activation via ATP release in sterile inflammation caused by acetaminophen (Hoque et al., 2012). Altogether, this literature, combined with our data presented here, may suggest a role for PXR activation to stimulate drug toxicity and sterile inflammation through NLRP3 inflammasome activation and extends the overarching implications of this thesis.
But beyond the potential involvement of PXR in drug toxicity, the data presented here suggests a potential role for the PXR as a link between pesticides within the environment and the observed inflammatory responses triggered by these environmental pollutants. Air pollutants have been shown to trigger innate immune activation through activation of various PRRs (Bauer, et al., 2011). More specifically, one study performed by Becker and others (2005) demonstrated that human airway epithelial cell exposed to air pollutants triggered increased TLR4 expression and IL-8 production and increased expression of oxidant stress response genes (Becker et al., 2005). Furthermore, an alternate study implicated pro-inflammatory cytokine and chemokine release in response to cigarette smoke in an in vitro dendritic cell model (Mortaz et al., 2009). To add to this, organochlorine pesticides (OCPs) have been demonstrated to induce ROS production within murine macrophages, triggering mitochondrial damage and ultimately apoptosis (Zhao et al., 2009). Overall, data indicate that pollutants trigger PRR expression, cytokine and chemokine release, and induce mitochondrial damage (Becker et al., 2005) (Mortaz et al., 2009) (Zhao et al., 2009).

PXR ligands are structurally and functionally diverse, and include a number of environmental pollutants such as polybrominated diphenyl ether flame retardants and OCPs (Zhang et al., 2008). One such study evaluating the effect of pollutants on PXR activation performed by Coumoul and others (2002) revealed that OCPs trigger PXR activation and increased CYP3A4 mRNA expression; this suggests that prolonged exposure to pollutants, such as pesticides, could activate a highly involved metabolic pathway within the liver through the PXR (Coumoul et al., 2002). However, beyond this, data collected from this study may provide a functional link between environmental pollutants and increased cytokine release noted with prolonged exposure to pollutants, as PXR activation in response to pollutants may trigger
inflammasome activation, causing IL-1β release and inducing inflammation (Zhang et al., 2008) (Becker et al., 2005). The potential broad reaching implications of findings presented in this thesis include a link between environmental pollutants and innate immune activation in the form of the xenobiotic receptor, PXR.

In this thesis, we have identified a new role for the PXR in triggering NLRP3 activation inflammasome. This PXR activation was shown to trigger NLRP3 activation through stimulating ATP release. Further examination of the role of PXR within macrophages may highlight other aspects of PXR function within immune cells and provide a deeper understanding into the workings of the NLRP3 inflammasome function. Overall, these data presented in this study may contribute to a greater understanding of NLRP3 inflammasome biology and may provide therapeutic targets for disease involving NLRP3 inflammasome dysfunction.

Image 4.0 Proposed mechanism of PXR induced NLRP3 inflammasome activation
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